

The Effect Of Nutrition And Metabolic Hormones On Follicular Development In Cattle

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ABSTRACT

Nutrition has profound effects on reproductive performance in cattle. Although these effects have been documented, the underlying mechanisms are not yet fully understood. The aim of this project was to investigate the role of metabolic hormones in mediating these nutritional effects by studying bovine folliculogenesis. The interaction of metabolic hormones and gonadotrophins on granulosa cell function *in vitro* was also investigated.

To determine the effect of nutrition on ovarian follicular development, twenty-eight heifers were allocated to different dietary treatments. Small follicle number (< 4 mm) was increased by 37% on days 1 and 2 of the oestrous cycle in heifers consuming twice maintenance requirements (2M). Insulin concentrations were higher in 2M than in control or feed-deprived heifers. The increase in small follicle number was independent of changes in peripheral FSH and insulin-like growth factor-I (IGF-I), negatively associated with growth hormone (GH) but positively associated with circulating insulin. Numbers of medium-sized (4 to 8 mm) and large (> 8mm) follicles and FSH concentrations were not different among treatments.

The relationship between postpartum follicular development and endocrine and metabolic changes in lines selected for high or low predicted breeding value for milk yield (PBV) was investigated. PBV was strongly correlated with milk production during a 305 day-lactation. This was associated with higher peripheral GH and β -hydroxybutyrate, and lower insulin and glucose concentrations in high genetic merit cows. Although no differences were seen in follicular development between selection lines, changes in body weight influenced follicle number, and day to first ovulation postpartum was delayed in cows selected for high milk production.

To gain an insight into the mechanisms underlying the nutritional effect on folliculogenesis, a serum-free bovine granulosa cell culture system was developed. In the presence of FSH, granulosa cells from small follicles differentiated *in vitro* and oestradiol (E_2) secretion increased with time. Cells from medium-sized and large follicles secreted E_2 throughout the culture period. Insulin and FSH promoted proliferation and E_2 production in a dose-responsive manner. The inclusion of IGF-I

enhanced proliferation and E_2 production, even in the absence of FSH. Furthermore, cultured granulosa cells formed clumps of spherical cells with ultrastructural characteristics that resembled those of granulosa cells *in vivo*. In contrast, granulosa cells growing either in the base of the clump, or as monolayers on serum-coated wells, possessed morphological characteristics suggestive of early luteinisation.

IGFs, as mediators of steroidogenesis and proliferation of granulosa cells, can act through endocrine, autocrine and/or paracrine mechanisms. Neither insulin nor FSH induced detectable levels of IGF-I production in granulosa cells, but IGF-binding proteins were secreted. IGF-I mRNA expression was found in theca cells, but not in granulosa cells either before or after culture. In contrast, IGF-II was expressed in both theca and granulosa cells.

The interaction of IGF-I and insulin on granulosa cell proliferation and E_2 production *in vitro* was determined in cells from follicles from cattle without or with a dominant follicle *in vivo*, i.e. ovaries were collected either on day 3 or day 7 of the oestrous cycle respectively. E_2 production and proliferative capacity of granulosa cells from medium-sized follicles *in vitro* was inhibited by the presence of a dominant follicle during their development *in vivo*. However, after long-term culture, E_2 production did not differ between granulosa cells either from day 3 or from day 7 follicles, but there were differences in proliferative responses to FSH and IGF-I according to the size of the follicle and the stage of the follicular wave.

The effects of FSH, EGF and IGF-I on early follicular development were studied by culturing preantral follicles. Follicle and oocyte diameter increased with time in culture. FSH, IGF-I and EGF stimulated follicle growth, but not oocyte growth rate. Most follicles maintained their morphology throughout culture and antra developed after 10 to 28 days of culture. EGF, IGF-I and FSH increased the probability of antrum development.

These results demonstrate that insulin and IGF-I, but not GH, may mediate the direct effects of nutrition on follicular development. IGFs may also have an autocrine action in the follicle, being tightly regulated by the production of IGF-BPs within the follicle.

DECLARATION

I hereby declare that this thesis has been composed by myself, and has not been submitted for any other degree, in Edinburgh or elsewhere. The work presented herein is my own, and all work of other authors is duly acknowledged. I also acknowledge all assistance given to me during the designing and execution of the experiments contained in this thesis and during its preparation.

Carlos Guillermo Gutierrez-Aguilar

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Gutierrez CG, Campbell BK, Gong JG, and Webb R. (1994) Bovine granulosa cell proliferation in vitro: the effect of the stage of the follicular wave. J. Reprod. Fertil. Abstr. Series. 14:Abstr. 22.

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Gutierrez CG, Campbell BK, Bramley TA and Webb R. (1995) Comparison of the ultrastructural characteristics of bovine granulosa cells cultured in the presence or absence of serum. J. Reprod. Fertil. Abstr. Series. 16:Abstr. 20.

Gutierrez CG, Gong JG, Baxter G, Bramley TA and Webb R. (1996) Dynamics of ovarian follicular cysts in postpartum dairy cattle. XIX World Buiatrics Congress. Edinburgh.

Gutierrez CG, Armstrong DG, Campbell BK, Hogg CO, and Webb R. (1996) Insulin-like growth factor (IGF) I production and expression of IGF-I and II by bovine granulosa and theca cells in vivo and in vitro. J. Reprod. Fertil. Abstr. Series. 17:Abstr. 66.

Gutierrez CG, Campbell BK, Gong JG, Bramley TA and Webb R. (1996) The effect of the stage of the follicular wave on bovine granulosa cell oestradiol production in vitro. J. Reprod. Fertil. Abstr. Series. 18:Abstr. 10.

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Introduction

Reproductive performance in cattle is a key determinant for efficient livestock production. Nutrition has a profound effect on reproductive performance. Although these effects have been widely documented, the underlying mechanisms are not yet fully understood. Variations in nutrition are paralleled by physiological adjustments reflected in changes in metabolic hormones which may act directly or in coordination with gonadotrophins to regulate follicular development. The aim of the work in this thesis was to investigate the role of metabolic hormones in mediating nutritional effects on bovine follicular development, and to investigate the interactions of metabolic hormones and gonadotrophins on granulosa cell function *in vitro*.

1. LITERATURE REVIEW

1.1 INTERACTIONS BETWEEN NUTRITION AND REPRODUCTION

The effect of nutrition on reproduction of livestock are well known. Evidence has been gathered since the time of Darwin. In *The origin of species* (Wade and Schneider, 1992), Darwin relates that domestic animals reproduce more efficiently than their wild ancestors, speculating that a readily available food supply was, at least in part, responsible for this effect. Downing and Scaramuzzi (1991), mentioned empirical observations from the first half of the last century, highlighting the effects of pasture quality and body condition in increasing the percentage of twin lambings.

Correlative data in wild animal species showed that they generally reproduced during the favourable time of the year where more nutrients were available. The same trend was seen in bovine species, a species that reproduces throughout the year (Aleksiev *et al.*, 1988). In tropical areas where seasonal variations in food quantity and quality are acute, reproductive performance of cattle is profoundly affected. Analysis of reproductive records of rangeland grazing cattle over 15 years found that calvings tended to cluster during the spring, indicating higher fertility during the summer months, when grass growth was at its maximal (Enriquez *et al.*, 1993). Moreover, Putu *et al.* (1986) found seasonal variation in fertility trials in buffaloes, though, when nutrient intake was standardised throughout the seasons the differences in fertility disappeared.

Other reproductive parameters are also known to be affected by deficient nutrition. Puberty can be delayed by halting the growth of the animals (see review by Robinson, 1990). Gonzales-Padilla *et al.* (1975) demonstrated that puberty in heifers could be delayed by restricting nutrition, and that heifers cycled synchronously after food was supplied *ad libitum*. Similarly, nutritionally growth-restricted lambs became hypogonadotrophic and this effect was reversed when feeding was re-established (Landefeld *et al.*, 1989; Foster *et al.*, 1989; Ober and Malven, 1992; I'Anson *et al.*, 1994). Similar observations have been made in other mammalian species (Wade and Schneider, 1992). These results support the theory developed by

Lamond (1970) that puberty is a function of live weight rather than age (Kaur and Arora, 1995). In cycling heifers, reduction of energy intake led to anoestrus when body weight fell by approximately 20% (Imakawa *et al.*, 1986a; Richards *et al.*, 1989a; Rhodes *et al.*, 1995). First ovulation postpartum (and thus calving interval) was prolonged by poor nutrition in both dairy (Whitaker *et al.*, 1993; Esteban *et al.*, 1994; Zurek *et al.*, 1995; Senatore *et al.*, 1996; Beam and Butler, 1997) and beef cattle (Short and Adams 1988; Dunn and Moss, 1992; Grimard *et al.*, 1995; Jolly *et al.*, 1995). Body energy reserves and therefore body condition score at the time of parturition, was the single most important determinant of the length of postpartum anoestrus in beef cattle (Wright *et al.*, 1992; Bishop *et al.*, 1994) and cows calving in poor body condition remained in anoestrus for a longer time. In contrast, although body condition may play a role in postpartum reproductive efficiency in dairy cattle, the extent of the negative energy balance, caused by the high demands of early lactation (Lucy *et al.*, 1991a; Veerkamp *et al.*, 1994) determined the interval from parturition to first ovulation postpartum (Butler and Smith, 1989; Swanson, 1989; Canfield and Butler, 1990). Follicular development in dairy cattle is re-established early during the postpartum period (Savio *et al.*, 1990; Beam and Butler, 1997). However, the first ovulation postpartum was delayed until the energy balance in animals had passed its nadir and energy balance although still negative, had begun to recover (Zurek *et al.*, 1995; Senatore *et al.*, 1996; Beam and Butler, 1997).

In conclusion, compared to thermoregulation, locomotion, growth, cellular maintenance and lactation, nutritional requirements for reproductive function occupy a low physiological priority (Short and Adams, 1988) when energy is restricted. Hence, reproduction is arrested well before other more vital functions are compromised (Dunn and Moss, 1992; Wade and Schneider, 1992).

1.1.1 THE EFFECT OF NUTRITION ON FOLLICULAR DEVELOPMENT

In cattle ovaries, regardless of their reproductive status, follicles in all stages of development are present. Moreover, antral follicular development proceeds in a typical wave-like pattern initiated by the growth of a number of small (2-5 mm)

follicles, from which only a handful escape atresia and grow beyond 4-5 mm in diameter. Finally only one large follicle ($< 8\text{mm}$) normally emerges as the dominant follicle (Driancourt *et al.*, 1987; Baird *et al.*, 1991; Fortune *et al.*, 1991; Fortune, 1994; Webb *et al.*, 1994; Campbell *et al.*, 1995). The dominant follicle present at the regression of the corpus luteum will induce the preovulatory peak of gonadotrophins, ovulate and luteinise. This normal pattern of follicular development has been shown to be altered by nutritional deficiencies.

In beef cattle, the diameter of the preovulatory follicle decreased when animals were losing weight (Murphy *et al.*, 1991; Grimard *et al.*, 1995; Rhodes *et al.*, 1995). Furthermore, the reduction in follicle diameter correlated positively with weight loss until the animals ceased to ovulate (Rhodes *et al.*, 1995). In addition, the oestradiol secretion of follicles in animals losing weight was half that of follicles from animals gaining weight (Spicer *et al.*, 1991). In the studies of Murphy *et al.* (1991) and Spicer *et al.* (1991), the number of follicles $> 5\text{mm}$ was not altered by body weight loss, although dominant follicles showed a decrease in diameter. However, reduction in the number of small ($<6\text{ mm}$) follicles was observed under more extreme undernutrition (Gutiérrez, 1992). In contrast, when heifers were gaining weight (Rhodes *et al.*, 1995; Murphy *et al.*, 1991; Spicer *et al.*, 1991) the diameter of the largest follicle increased and the follicle appear to be dominant, as the number of subordinate follicles was reduced as the first ovulation approached (Gutiérrez *et al.*, 1994).

Dairy cattle immediately after parturition suffer negative energy balance. This negative energy balance is caused by the large energy demands of lactation, together with low appetite in the immediate postpartum period (Butler and Smith, 1989; Swanson, 1989). Waves of follicular development in postpartum cows are normally initiated early after calving, following an increase of plasma FSH within the first 5 days postpartum (Beam and Butler, 1997). During this period, the extent of the energy balance deficit affects follicular development. Compared with non-lactating cows (De la Sota *et al.*, 1993), lactating cows had a lower energy balance, reflected in lower plasma concentrations of glucose, insulin and IGF-I, but higher

concentrations of non-esterified fatty acids (NEFA). The lower energy balance of the lactating cows was accompanied by fewer follicles of < 15 mm in diameter (De la Sota *et al.*, 1993) compared to non-lactating cows, indicating that low energy balance decreased the number of small follicles progressing into the large diameter categories. However, in other studies, lower energy balance in lactating cows was associated with higher numbers of small (3-5 mm) and medium-sized (6-9 mm) follicles (Lucy *et al.*, 1991a,b). Furthermore, when cows were fed low or high energy diets, the growth rate of preovulatory follicles in low energy diet cows was slower than that in cows on a high energy diet (Murphy *et al.*, 1991; Lucy *et al.*, 1992).

Paradoxically, treatment of lactating cows with bovine somatotrophin (BST; which increases milk production causing a decline in energy balance), stimulated an increase in the number of follicles reaching larger size categories, with a consequent decline in small (<5 mm) follicle number (De la Sota *et al.*, 1993; Lucy *et al.*, 1993b). The explanation for the increase in the number of follicles progressing to larger follicle size categories is not clear, but could be due to changes in circulating concentrations of metabolic hormones and metabolites (*i.e.* insulin, IGF-I, IGF-binding proteins, glucose) induced by growth hormone in lactating cows (Pell and Bates, 1990; Bauman, 1992; De la Sota *et al.*, 1993).

Although dominant follicles appear in the ovaries of lactating cows early in the postpartum period (Savio *et al.*, 1990; Beam and Butler, 1997), the functional capability of these dominant follicles might be altered by low energy balance. Lucy *et al.* (1992) found that the oestradiol to progesterone ratio in the follicular fluid of dominant follicles was lower in cows fed low energy diets, possibly suggesting that these follicles do not reach the final maturational stage. Hence this could explain the failure of the dominant follicle to trigger the preovulatory peak of LH (Hansel and Convey, 1983), thereby resulting in an extended period of postpartum anoestrus, even though follicular waves still occur. In support of this it has been shown that the number of follicular waves where the dominant follicle did not ovulate was positively correlated with the time taken for energy balance to reach its nadir (Canfield and Butler, 1990; Zurek *et al.*, 1995; Senatore *et al.*, 1996; Beam and

Butler, 1997). In addition, the dominance mechanism seems to be affected by energy balance. Indeed, lower dietary energy and BST treatment were both associated with larger subordinate follicles (Lucy *et al.*, 1993b). Furthermore, suppression of the growth of small and medium-sized follicles is more efficient as energy balance becomes less negative (Lucy *et al.*, 1991a)

Nutrition may also have positive effects on reproductive performance. One of the best known examples is perhaps the increase in litter size provoked by additional nutritional supply for a short time prior to mating (*i.e. flushing*) (Robinson, 1990; Downing and Scaramuzzi, 1991; Dunn and Moss, 1992; Kaur and Arora, 1995). This effect is well recognised in sheep, goats and pigs (Robinson, 1990). However, in these species, due to the scarcity of longitudinal studies on follicular development *in vivo* (Souza *et al.*, 1996), the effects of nutrition have only been noted on ovulation rate and litter size. Nonetheless, from studies where ovaries were recovered after different nutritional treatments, it was noted that flushing or infusion of insulin or glucose decreased the percentage of atretic follicles in the ovaries (cattle, Maurase *et al.*, 1985; pigs, Matamoros *et al.*, 1990; 1991). In conclusion, although our understanding of the role of nutrition on reproductive performance has advanced considerably, we still do not know the mechanisms of action.

1.1.1.1 Possible mechanisms for the effects of nutrition on follicular development

From the evidence discussed above, it is clear that nutrition has major effects on reproduction that could be either detrimental, caused by nutritional deficiencies and reducing reproductive performance, or beneficial, associated with higher body weight, positive energy balance and/or flushing (a period of enhanced feeding, particularly in sheep). In this section the mechanisms of action of both detrimental and beneficial effects of nutrition on follicular development are reviewed.

Other effects of nutrition on reproductive performance are caused by nutrient excesses (see Dunn and Moss, 1992). However, they will not be reviewed in this thesis.

1.1.1.2 Effect of nutrition on FSH, LH and GnRH secretion

Follicular development is primarily controlled by the co-ordinated action of gonadotrophins (McNeilly *et al.*, 1991; Campbell *et al.*, 1995). The gonadotrophin requirements for follicular growth in cattle were recently determined by Gong *et al.* (1995; 1996a), who used long-term treatment with a GnRH-agonist to suppress LH release (Gong *et al.*, 1995) or both LH and FSH release (Gong *et al.*, 1995; 1996a) from the pituitary gland. They showed that without pulsatile release of LH, follicle development could proceed, but was halted when the dominant follicle reached 7-9 mm in diameter. When FSH concentrations were suppressed after a long period of GnRH-agonist treatment follicle growth stopped at 4 mm in diameter (Gong *et al.*, 1996a). Therefore, since follicle development depends on sustained support by gonadotrophins, changes in gonadotrophin secretion, caused by nutrition, may affect follicular development.

Circulating FSH concentrations in intact animals are not affected by nutrition. Rhodes *et al.* (1995) found no changes in FSH concentrations in undernourished heifers even after they had lost 17% of their body weight. Similarly, Rhind *et al.* (1989) did not observe changes in FSH concentrations in ewes with high or low body condition scores. However, failure to observe differences in FSH concentrations in the previous studies could have been due to the ovarian regulation of FSH secretion (Baird *et al.*, 1991). Indeed, ovariectomised animals in good nutritional status had higher FSH circulating concentrations than those in poorer nutritional status (Rhind *et al.*, 1989; Foster *et al.*, 1989; Landefeld *et al.*, 1989).

Although some studies were unable to find differences in the pattern of LH secretion in intact ewes (Rhind *et al.*, 1989; Albecia *et al.*, 1995) or heifers (Rhodes *et al.*, 1995; Enright *et al.*, 1994), there seems to be a consensus that significant changes in

body weight or nutrient supply can alter the pattern of LH secretion. This was shown first by Gonzales-Padilla *et al.* (1975) and corroborated by Yelich *et al.* (1996) in feed-restricted heifers, and by Foster *et al.* (1989) and Landefeld *et al.* (1989) in feed-restricted ewes. In these studies, feed-restricted animals were maintained in a prepubertal state due to suppressed mean concentrations of LH and LH pulse frequency. However, after improvement of the diet, there was an increase in mean concentrations and pulse frequency of LH. Similar responses to improved feed intake have been found in studies with animals in diverse reproductive states, for instance in feed-restricted anoestrous heifers (Imakawa *et al.*, 1986b; McCann and Hansel, 1986; Richards *et al.*, 1989a), or ovariectomised sheep (Rhind *et al.*, 1989; Estienne *et al.*, 1990; Roberson *et al.*, 1991; Roberson *et al.*, 1992; Wright *et al.*, 1992). In beef cows the body condition score (BCS) at parturition was directly related to LH pulse frequency after weaning, but LH pulsatility was not influenced by time after weaning (Bishop *et al.*, 1994). In dairy cows, Lucy *et al.* (1991b) found no effect of diet on LH secretion, probably because their samples were taken too close to the nadir in energy balance. However, Canfield and Butler (1990) observed that baseline and mean concentration of LH and LH pulse frequency were higher in cows after the energy balance nadir compared to before the energy balance nadir. Consequently, nutrition appears to affect pituitary gonadotrophin secretion, and LH pulse frequency in particular, preventing the final maturation of a potentially ovulatory follicle.

However, in most cases the pituitary gland is capable of releasing substantial amounts of LH and FSH after GnRH stimulation, and the amount of LH released in response to GnRH does not change when heifers with initial high BCS were losing weight. However, the LH response to GnRH was reduced in heifers with an already low BCS when they lost weight further (Roberson *et al.*, 1992). Furthermore, Landefeld *et al.* (1989) found that the pituitary content of LH did not change with diet, although FSH pituitary content did increase in well fed lambs. Messenger RNA levels for α -subunit, LH β and FSH β increased with increased nutrition (Landefeld *et al.*, 1989). On the other hand, LH and FSH receptor levels in ovarian tissue were not altered by level of nutrition (Rhind *et al.*, 1992; Albecia *et al.*, 1995). Taken together these results indicate that the response to GnRH is reduced once a compromising

BCS is reached. Secondly, synthesis and storage of gonadotrophins, or sensitivity response of the pituitary gland to GnRH, are reduced when BCS is low.

In conclusion, FSH concentrations in intact animals are not acutely influenced by nutritional status. However, LH concentrations and pulse frequency appeared to decrease when animals were restricted to sub-maintenance nutrition.

1.1.1.3 Role of metabolic hormones on follicular development

It was soon recognised that factors other than gonadotrophins may affect the regulation of follicular development. Enhanced follicular development and ovulatory responses obtained with flushing treatments were independent of changes in circulating concentrations of FSH. Therefore, the mechanisms regulating the increase in follicle number must lie with factors distinct from those of the hypothalamo-pituitary-ovarian axis. The role of metabolic hormones in mediating the effects of nutrition on reproduction was evident from the positive correlation between their circulating concentrations and nutritional changes. Further evidence for the involvement of metabolic hormones other than gonadotrophins, in enhancing follicular development comes from studies in women with polycystic ovarian syndrome (PCOS). This syndrome is associated with hyperinsulinaemia and impaired glucose tolerance (Diamanti-Kandarakis and Dunaif, 1996; Poretsky, 1991).

1.1.1.3.1 Role of the somatotrophic axis on follicular development

1.1.1.3.1.1 Growth hormone (GH)

Growth hormone is a pituitary hormone, secreted in an episodic fashion under the control of the hypothalamic peptides GH-releasing hormone (GH-RH) and somatotrophin releasing inhibitory factor (SRIF) (Dieguez and Casanueva, 1995; Clark and Robinson, 1996). GH-RH stimulates synthesis of GH in the pituitary gland and has trophic action in the pituitary somatotroph cells (Clark and Robinson, 1996). The release of GH from the pituitary gland is also under the control of somatostatin

(SRIF), a hypothalamic hormone that blocks GH-RH stimulated GH release. SRIF blocks the release of GH. Hence some pulses of GH-RH are not followed by GH release. Therefore, for a GH pulse to occur, a GH-RH pulse must coincide with a decline in SRIF concentrations. Furthermore, GH-RH-independent pulses of GH may occur when SRIF secretion declines (Clark and Robinson, 1996).

Circulating concentrations of GH are negatively correlated with nutritional status. In general, GH concentrations in sheep (Foster *et al.*, 1989; Landefeld *et al.*, 1989; Clarke *et al.*, 1993; Cole *et al.*, 1993), cattle (Breier *et al.*, 1986; Ropke *et al.*, 1994), deer (Webster *et al.*, 1996) and humans (Pell and Bates, 1990; Rodgers, 1996) increase during catabolic conditions such as under-feeding, whereas GH concentrations are decreased in overfed or obese individuals.

Dieguez and Casanueva (1995) recently reviewed the most likely nutritional regulators of GH secretion. Plasma glucose concentrations are major determinants of GH secretion. Hence, acute hyperglycaemia inhibits GH secretion, whilst hypoglycaemia is one of the most potent stimulators of GH secretion. Furthermore, the effects of glucose on GH secretion are not regulated by insulin (Murao *et al.*, 1994) and changes in GH release are independent of hypothalamic changes in GH-RH. Therefore, the most likely scenario is that changes in GH secretion induced by nutrition or obesity are the result of changes in hypothalamic secretion of SRIF (Murao *et al.*, 1994; Thissen *et al.*, 1994; Dieguez and Casanueva, 1995). In addition, a decline in circulating GH concentrations can be caused by GH-stimulated hepatic IGF-I (Pell and Bates, 1990; Simmen, 1991).

GH action is further regulated by the amount of GH-binding protein (GH-BP) in the circulation. GH-BP corresponds to the extracellular domain of the GH receptor, which, when associated with GH, reduces the metabolic clearance rate of GH (Pell and Bates, 1990; Clark and Robinson, 1996). In addition, GH-BP levels are also regulated by nutrition, since improved nutrition causes an increase in circulating GH-BP, whereas fasting or mal-nutrition decreases the amount of circulating GH-BP (Barnard and Waters, 1997). Furthermore, variations in GH-BP levels parallel changes in GH receptor expression *in vivo*. Consequently, GH-BP increases GH half-

life, whilst decreasing GH bio-availability. Thus potentiation or inhibitory effects of nutrition might depend on the relative concentrations of GH, GH-BP and GH receptor, although detailed experimental information is lacking at the moment.

In women, GH has been used in combination with gonadotrophins to improve the response and reduce the doses of gonadotrophin required in *in vitro* fertilisation (IVF)-ovarian stimulation programmes. Some investigators support the contention that GH enhances the ovarian response to gonadotrophin (Jacobs, 1993) and reduces the doses of gonadotrophins needed (Homburg *et al.* 1990), although this view is not universally shared (Filicori, 1993). It appears that co-administration of gonadotrophins and GH is of benefit in patients with hypogonadotrophic hypogonadism, PCOS and human menopausal gonadotrophin-resistance.

In animals, pharmacological administration of GH to heifers increased the number of small follicles (<5 mm), but did not alter the number of follicles (>5 mm) or the dynamics of follicle turnover (Gong *et al.*, 1991;1993a). Furthermore, GH increased follicle number in the absence of changes in circulating concentrations of FSH and LH, and gonadotrophin binding to theca and granulosa cells (Gong *et al.*, 1991). Similarly, in dairy cows recombinant GH treatment increased the number of follicles without altering gonadotrophin levels (De la Sota *et al.*, 1993). However, increased follicle numbers were seen in the larger size categories (>5 mm) (De la Sota *et al.*, 1993; Lucy *et al.*, 1993b), and in one study (Kirby *et al.*, 1997) follicular turnover was enhanced by earlier onset of atresia of the first dominant follicle. Growth hormone treatment in other species (pigs, Spicer *et al.*, 1992a; rabbit, Yoshimura *et al.*, 1994; sheep, Gong *et al.*, 1996b) also increases the number of antral follicles. In addition, Gong *et al.* (1993b) showed that the small (<5 mm) follicles induced by GH stimulation were responsive to exogenous gonadotrophin stimulation, thus increasing the response to superovulatory treatments compared to heifers not treated with GH. Moreover, low GH levels may also affect follicular development. Cohick *et al.* (1996) immunised prepubertal heifers against GH-RH, thereby reducing the circulating concentrations of GH, and found that only 22% of heifers developed follicles above 7mm in diameter, compared with 77% of control heifers. In contrast,

Adams *et al.* (1996) did not observe changes in follicular growth or gonadotrophins levels in GH-RH immunised adult sheep. These results taken together indicate that GH plasma concentrations influence follicular development *in vivo*. However, the follicle size category affected by GH appears to vary, perhaps due to the differential responses of IGF-I and insulin secretion to GH treatment *in vivo*, according to the physiological status of the animal (see below).

In vitro, recombinant GH did not stimulate proliferation or steroidogenesis of bovine granulosa cells from follicles <10 mm in diameter (Gong *et al.*, 1993c; 1994). However, in granulosa cells derived from follicles >10mm in diameter, GH stimulated proliferation and oestradiol and progesterone production. Similarly, Wathes *et al.* (1995) reported that GH stimulated progesterone production by ovine preovulatory follicle granulosa cells after 4 days of culture. Spicer and Stewart (1996b), found that GH had no effect on steroidogenesis of bovine granulosa cells, though high doses (300 ng/ml) of GH inhibited oestradiol production. Furthermore, GH stimulated oxytocin production by bovine granulosa cells from small follicles (2-5 mm) in a culture system containing 10% serum (Sirotkin and Nitray, 1995). In thecal cells, GH stimulated androstenedione production in LH responsive cultures (Spicer and Stewart, 1996a), but in cultures where thecal cells did not respond to LH stimulation, GH inhibited androstenedione production. In addition, direct GH administration into the ovarian artery did not stimulate ovarian steroid secretion in ovarian autotransplanted sheep (Campbell *et al.*, 1995).

In summary, GH does not affect proliferation or steroidogenesis of granulosa cells from immature follicles, but increased steroidogenesis in response to GH in cells from large follicles, which is perhaps linked to the degree of cell differentiation in culture. Moreover, the response of both granulosa and theca cells to GH *in vitro* appeared to vary depending on time in culture and gonadotrophin responsiveness of the cells. Thus it is most likely that GH responsiveness increases with the degree of cell luteinisation. Further support for this is the lack of GH receptor, or its mRNA, in bovine follicles (Lucy *et al.*, 1993a), though GH receptor and its mRNA were highly

expressed in luteinised cells (Lucy *et al.*, 1993a; Kirby *et al.*, 1996). Thus, a direct effect of GH on follicular development is still not proven.

In vivo, GH has systemic effects orchestrated to channel nutrients towards specific physiological processes (for reviews see Pell and Bates, 1990; Bauman, 1992). Growth hormone increases gluconeogenesis, thus augmenting the circulating concentration of glucose and thereby increasing insulin. At the same time, GH causes insulin resistance and insulin-antagonist effects reducing liponeogenesis and increasing lipolysis. These changes divert nutrients from fat deposition towards the target processes (*i.e.* growth, lactation, convalescence). Growth hormone directly stimulates the production of IGF-I by the liver, which mediates most of the growth-promoting effects of GH (Rodgers, 1996). Thus, GH administration *in vivo* results in increased circulating concentrations of insulin, IGF-I and glucose (Gong *et al.*, 1993a; De la Sota *et al.*, 1993; Roeder *et al.*, 1994). Therefore, although GH might not affect follicular development, it may indirectly modulate follicular development.

1.1.1.3.1.2 Insulin-like growth factor (IGF) system

IGF-I and -II are small insulin-like peptides which have mitogenic and differentiative functions in a wide range of tissues. IGF-I is thought to mediate most of the actions of GH (Lund, 1994; Jones and Clemmons, 1995). In contrast, IGF-II is believed to be the major regulator of prenatal growth (Giudice, 1992; Lund, 1994; Hossner *et al.*, 1997). IGF-I and -II activity is present in plasma samples at a molecular mass greater than 50 kDa, but can be converted to smaller forms by low pH treatment of plasma (Baxter and Martin, 1989). The demonstration of specific plasma binding sites for IGFs explained this phenomenon, and showed that two peaks of IGF immunoreactivity were present in acidified samples after size-exclusion acid chromatography (Hints and Liu, 1977). The first peak corresponded to high affinity IGF binding sites (IGFBP) and the second one contained the bioactive peptides. However, reassociation of the peptide and the binding proteins occurs when samples

are neutralised (Hints and Liu, 1977). Although the functions of IGFBPs are not yet fully understood, they appear to serve in IGF transportation, prolongation of the half-life of IGFs (Lund, 1994; Jones and Clemmons, 1995), as extracellular storage sites of IGF (Logan and Hill, 1992) and possibly mediate the interaction between IGF and its receptor (Jones and Clemmons, 1995). Recently, data suggest that IGFBPs may also have actions which are independent of IGFs (Rosenfeld *et al.*, 1997).

1.1.1.3.1.2.1 IGFs.

The main source of IGF-II is the liver. Serum IGF-II concentrations appear to be independent of GH or nutritional variations (Giudice, 1992; McGuire *et al.*, 1992). In contrast, serum IGF-I is produced primarily in the liver in response to GH stimulation and as for GH, IGF-I levels are affected by nutritional status. Indeed, feed restriction provokes a decline in circulating levels of IGF-I, despite elevated GH concentrations (Breier *et al.*, 1986; Armstrong *et al.*, 1993; Clarke *et al.*, 1993; Yambayamba *et al.*, 1996). However, after normalisation of feed intake, circulating IGF-I concentrations are also normalised (Thissen *et al.*, 1994; Yambayamba *et al.*, 1996). Exogenous GH, provokes an increase in circulating concentrations of IGF-I in well-fed individuals. However, in underfed animals, the GH-induced rise in IGF-I was absent (Elsasser *et al.*, 1989; Hua *et al.*, 1995; McGuire *et al.*, 1992).

The mechanism by which under-nutrition reduces IGF-I secretion seems to involve insulin. When insulin concentrations are low in underfed animals, there is a decline in GH receptor capacity (Pell and Bates, 1990; McGuire *et al.*, 1992; Thissen *et al.*, 1994), with GH resistance. The administration of insulin seems to reverse this effect. Moreover, dietary components seem to determine the basal amount of IGF-I secreted by the liver. Elsasser *et al.* (1989) found that IGF-I concentrations were positively correlated with the amount of protein in the diet, once energetic requirements of steers were fulfilled. Furthermore, liver from fasted rats and aminoacid-deprived hepatocytes had decreased IGF-I mRNA expression and reduced IGF-I transcription (Hayden *et al.*, 1994). However, when insulin or aminoacids were administered,

transcription increased (Pao *et al.*, 1993). These results indicate that protein and insulin regulate the circulating concentrations of IGF-I, and that nutrition regulates IGF-I gene expression primarily at the transcriptional level.

Oestrogen is a potent regulator of IGF-I biosynthesis, and IGF-I mRNA expression is enhanced by oestradiol (Simmen, 1991). This may explain the retarded growth seen in hypogonadotrophic patients with Turner's syndrome.

IGF-I is a potent stimulator of steroidogenesis and proliferation of both granulosa and theca cells *in vitro*. IGF-I stimulated granulosa aromatase activity and granulosa cell number (sheep, Campbell *et al.*, 1996a; cattle, Spicer *et al.*, 1993b; pigs, Howard and Ford, 1994; rats, Adashi, 1992b) and androstenedione production by theca cells (Caubo *et al.*, 1989) *in vitro*. In addition, IGF-I analogues, either des-(1-3)-IGF-I or LR3-IGF-I, were more potent in stimulating the response of granulosa cells than the native form of the hormone (Howard and Ford, 1994). This was presumably because the analogues have reduced affinity for the IGF-I binding proteins (IGF-BP) produced by granulosa cells in culture (Armstrong *et al.*, 1996b,c). The local production of IGF-BP by cultured cells might also explain why some reports found either no effect (Spicer and Stewart, 1996b) or inhibitory effects (Spicer and Echtenkamp, 1995) of IGF-I in cultured cells.

The way in which IGF-I stimulates cell proliferation (Guidice, 1992; Gong *et al.*, 1993c; Monget *et al.*, 1993; Jones and Clemmons, 1995; Campbell *et al.*, 1996a) and differentiation (Adashi, 1992a; Adashi and Rohan, 1992; Guidice, 1992; Gong *et al.*, 1994; Kanzaki *et al.*, 1994; Jones and Clemmons, 1995; Campbell *et al.*, 1996a) has been hypothesised to be by serving as an amplifier for gonadotrophic action (Adashi, 1992a; Adashi and Rohan, 1992; Kanzaki *et al.*, 1994). In the sheep, IGF-I has additive effects with insulin and FSH to enhance oestradiol production and proliferation *in vitro* (Campbell *et al.*, 1996a).

Few reports are available on the effects of IGF-I *in vivo*. Indirect evidence of the effects of IGF-I on follicular development *in vivo* comes from studies where BST, which increases circulating IGF-I, was used to enhance follicular development (Gong *et al.*, 1993b,c). In sheep, Campbell *et al.* (1995) infused LR3-IGF-I directly into the

ovarian artery, provoking an acute and prolonged stimulation of ovarian steroid secretion. Further proof of the involvement of IGF-I in ovarian function is that mice produced by IGF-I gene knockout, were hypogonadal and sterile (Baker *et al.*, 1993; Nishimori and Matzuk, 1996).

In contrast to IGF-I, only a few reports on the effects of IGF-II on follicular function are available. IGF-II was found to inhibit granulosa cell oestradiol production and stimulate progesterone production (Spicer and Echternkamp, 1995). However, Caubo *et al.* (1989) found a stimulatory effect of IGF-II on androstenedione production of ovine theca cells, and synergy with hCG. The relative potency of both IGF-I and -II in steroidogenesis and proliferation of ovine granulosa cells, was compared using IGF analogues, which avoid interference by IGF-BPs (Campbell, 1997). The results showed differential stimulation of proliferation and differentiation by IGFs. Whilst IGF-I was a more potent stimulator of proliferation, IGF-II was found to preferentially stimulate steroidogenesis (Campbell, 1997).

The potent effects IGFs on proliferation and steroidogenesis of follicular cells made them good candidates to regulate processes such as recruitment, selection and dominance of follicular development. However, the relationship between follicular development and IGF-I or -II concentrations under physiological conditions has been more elusive. Efforts to detect variations in IGF-I concentrations during either the menstrual (Van Dessel *et al.*, 1996) or oestrous cycles (Spicer *et al.*, 1988; Funston *et al.*, 1995; Leeuwenberg *et al.*, 1996) have failed to show changes in IGF-I concentrations throughout the oestrous cycle. Echternkamp *et al.* (1990) found that cattle selected for twinning had higher concentrations of IGF-I in serum and follicular fluid than monotocous cattle. However, Spicer *et al.* (1993a) did not find an association between serum IGF-I concentration with ovulation rate in sheep, but reported that IGF-I was higher during the follicular phase of the oestrous cycle. Furthermore, IGF-I concentrations did not vary in follicular fluid from different sized follicles, between oestrogen-active and inactive follicles, with degree of atresia, and before and after GnRH administration (Spicer *et al.*, 1988; 1991; 1992b; Monget *et al.*, 1993; Stewart *et al.*, 1996). In addition, IGF-I concentrations in follicular fluid

did not differ from those in peripheral blood (Echternkamp *et al.*, 1990; Monget *et al.*, 1993; Leeuwenberg *et al.*, 1996) or ovarian-vein concentrations (Spicer *et al.*, 1993a). Thus the ovary does not appear to be a major source of circulating IGF-I.

Peripheral IGF-II also did not vary during the menstrual cycle in women (Van Dessel *et al.*, 1996). In the same study, Van Dessel *et al.* (1996) reported that IGF-II correlated directly with oestradiol content and diameter of the ovulatory follicle. However, in cattle, IGF-II concentration was higher in small than in large follicles (Spicer *et al.*, 1992b) and did not differ with stage of atresia (Stewart *et al.*, 1996).

1.1.1.3.1.2.2 *IGFBPs*

Six IGF-BPs have been characterised so far (Drop *et al.*, 1991; Jones and Clemmons, 1995; Hossner *et al.* 1997), with affinities for IGF equal to or higher than the IGF-I type-1 receptor (Logan and Hill, 1992). Recently, proteins sharing certain homology (20-25%) to IGFBPs (IGFBP-7, -8 and -9) have been characterised (Oh *et al.*, 1996; Rosenfeld *et al.*, 1997). Most of the peripheral IGFs are bound to IGF-BP3, and circulate as a ternary complex formed by IGF, IGF-BP3 and an acid labile subunit (ALS). IGF-BP3 levels are relatively constant in the circulation and it serves as the main store of IGF.

Binding proteins are regulated by nutritional status. IGFBP-3 and ALS are mainly produced in the liver and are GH dependent (Hossner *et al.*, 1997). As for IGF-I, animals on a high plane of nutrition have increased IGFBP-3 and ALS, whilst undernutrition decreases both IGFBP-3 and ALS. Furthermore, GH increases IGF-BP3 in well fed but not in undernourished sheep (Hodgkinson *et al.*, 1991). In contrast to IGFBP-3, IGFBP-1 and -2 increased in underfed animals (Pell and Bates, 1990; McGuire *et al.*, 1992), an effect that seems to be regulated by insulin (Pao *et al.*, 1993). Moreover, nutritional regulation of IGFBPs may be at both the transcriptional and/or translational level in the liver. Food deprivation in the rat decreased circulating IGFBP-3 and -4, without changes in their mRNA levels. In contrast, IGFBP-1 and -2 (which increased during food restriction) showed increased

transcription of their respective genes (Takenaka *et al.*, 1996). These coordinated changes in IGFBPs ensure that the correct amount of IGF reaches the target tissue, an action that was well illustrated recently by Huang *et al.* (1997). They found that transgenic mice overexpressing IGFBP-1 had a decreased litter size due to a reduction in the number of ovulatory follicles.

Peripheral concentrations of IGFBPs do not change during the cycle (sheep, Spicer *et al.*, 1993a; women, Van Dessel *et al.*, 1996). Thus there is little variation in peripheral IGF available from the circulation. However, variations in the local production of IGFBPs, or degradation of IGFBPs by proteolysis, may produce changes in IGF availability within follicles during follicle differentiation and atresia (Monget *et al.*, 1993; Armstrong *et al.*, 1996a,b; De la Sota *et al.*, 1996).

In conclusion, IGFs have potent stimulatory effects on a range of follicular functions. However, the different fate of follicles under the same gonadotrophin stimulus cannot be explained by the changes in circulating IGF-I concentrations at the ovarian level.

The large variation in the results reported, make it difficult to understand why follicles have different fates even when under same gonadotrophic stimuli. The variability in the results on the effects of IGF-I in most *in vivo* studies might be due to the interfering effects of IGFBPs when IGFs were measured using extraction procedures which fail to completely remove binding proteins from the samples (see Chapter 3). Unfortunately, reports where IGF-I has been measured after complete removal of binding proteins (*i.e.* acid chromatography) are not available to date. Alternatively, the action of IGF-I systemically or locally may be controlled by the regulation of IGFBPs.

1.1.1.3.2 Role of insulin and glucose in follicular development

Circulating concentrations of insulin and glucose are physiologically linked and directly correlated. The importance of insulin as a regulator of ovarian function is revealed in patients with altered insulin concentrations. Insulin-dependent diabetes mellitus in women is commonly accompanied by ovarian hypofunction and

amenorrhea, whilst hyperinsulinaemia is associated with ovarian hyperstimulation and hyperandrogenism (Poretsky and Kalin, 1987; Poretsky, 1991; Fulghesu *et al.*, 1997). In cattle, early studies (McClure, 1968) showed that hypoglycaemia induced by insulin on day 17 to 20 of the oestrous cycle delayed oestrus. Similarly, if glucose metabolism was blocked by 2-deoxy-D-glucose (McClure, 1978) on the day of expected oestrus, oestrus and corpus luteum formation were prevented. Thus, insulin and/or glucose levels modulate the regulation of follicular events, either at the hypothalamus-pituitary level, by regulating gonadotrophin secretion, or by acting directly on the ovary, or both.

High insulin concentrations in non-lactating heifers were associated with increased follicle (<4mm) number (Gong *et al.*, 1993a) and increased responses to superovulatory treatment (Gong *et al.*, 1993b). However, although FSH concentrations did not change, there was a concomitant increase in IGF-I and GH levels in the circulation. Administration of insulin alone at the time of a superovulatory regime of FSH (Simpson *et al.*, 1994) did not affect the number of follicles, but treated cows had larger and more oestrogenic follicles than control cows.

Glucose infusion in sheep (Downing *et al.*, 1995) increased insulin concentrations and ovulation rate without altering LH secretion. Although FSH concentrations were lower than in control ewes, this could have been due to the down-regulation of FSH caused by increased follicular development and the subsequent negative feedback of oestradiol and/or inhibin. However, in a second study in sheep (Downing and Scaramuzzi, 1997), insulin infusion failed to enhance ovulation rate. However in this later study, glucose concentrations were reduced to below 40% of pre-treatment levels and this may have caused a reduction in LH pulse frequency. In contrast, insulin infusion into pigs also at a dose causing 40% reduction in glucose concentrations, provoked an increase in follicle number, with an associated reduction in the number of atretic follicles (Matamoros *et al.*, 1990; 1991), without affecting FSH or LH concentrations, or hCG binding to granulosa cells (Matamoros *et al.*, 1991).

The effects of insulin deprivation on follicular development were studied in diabetic gilts where insulin replacement therapy had been withdrawn. Diabetic gilts have low insulin and IGF-I, but high glucose concentrations. Insulin withdrawal did not affect FSH concentrations or pulsatile LH secretion (Meurer *et al.*, 1991), but the percentage of atretic follicles increased (Meurer *et al.*, 1991; Edwards *et al.*, 1996).

Insulin does not appear to affect the control of gonadotrophin secretion. Also intra-cerebro-ventricular administration of insulin to feed-restricted sheep did not alter LH secretion, but when administered to sheep fed *ad libitum*, insulin decreased both LH pulse frequency and mean LH concentration (Hileman *et al.*, 1993). In contrast, when glucose (Bucholtz *et al.*, 1996) and fatty acid (Hileman *et al.*, 1991) metabolism was blocked, by the use of glucose or fatty acid non-metabolisable analogues, LH pulse frequency and mean LH concentration declined due to a reduction in GnRH secretion, though insulin concentrations were unchanged. These results, together with the fact that low insulin and high glucose levels in the diabetic gilts did not alter LH secretion (Meurer *et al.*, 1991; Edwards *et al.*, 1996) suggest that, at the central level, oxidisable fuel availability is a stronger signal than insulin concentration for controlling LH secretion.

In vitro, insulin has repeatedly been reported to stimulate proliferation and steroidogenesis of granulosa and theca cells (Campbell *et al.*, 1995; Spicer *et al.*, 1995) and is routinely included in tissue and cell culture media. Duleba *et al.* (1993) investigated whether the effect of insulin on follicular cells was mediated by glucose metabolism. They treated cultured rat granulosa and theca cells with metformin (an insulin agonist used for the treatment of non-insulin-dependent diabetes mellitus, that potentiates the effect of insulin on glucose metabolism at the post-receptor level), and found no augmentation of the insulin-stimulated steroidogenic response. This indicated that the modulatory effect of insulin on steroidogenesis is independent of glucose metabolism. Furthermore, when bovine theca cells were cultured in glucose-deficient medium (Spicer *et al.*, 1995), the addition of glucose or insulin alone stimulated proliferation and steroid synthesis and, when added together, their effects were additive.

Taken together, it appears that glucose, but mainly insulin concentrations, stimulate follicular development and steroidogenesis at the ovarian level, whereas centrally, glucose appears to be more important in controlling the secretion of gonadotrophins.

1.1.1.3.3 Leptin

It was first postulated (Lamond, 1970; Frisch and McArthur, 1974) that a critical amount of body fat was needed for the onset and/or maintenance of oestrous and menstrual cyclicity. However, this theory was dismissed in 1991 (Bronson and Manning, 1991) as no metabolic/neuroendocrine pathway linking fat stores and GnRH secretion was known.

Recently, the original concept of fat as a determinant of ovarian cyclicity has regained credibility with the discovery of leptin. Leptin is produced by white adipocytes with a putative action in regulating food intake (Billington and Levine, 1996; Weigle and Kuijper, 1996; White and Tartaglia, 1996; Friedman, 1997). In addition, circulating leptin concentrations increased in direct relation with body fat reserves (Boden *et al.*, 1996; Spiegelman and Flier, 1996). However, in the short-term, leptin concentrations are regulated by changes in circulating concentrations of glucose/insulin (Boden *et al.*, 1996). Therefore, leptin acts as a mediator between nutritional status and appetite centres in the brain to regulate food intake in both the long- and short-term.

Leptin deficient (ob/ob) mice are sterile and hypogonadotrophic (Swerdloff *et al.* 1976). In addition, administration of leptin to both male and female ob/ob mice, reduced food intake and body weight, increased circulating concentrations of gonadotrophins and restored fertility (Barash *et al.*, 1996; Chehab *et al.*, 1996; Mounzih *et al.*, 1997). Furthermore, leptin administration to prepubertal normal mice, reduced food intake and growth rate, but advanced puberty, with mice reaching puberty at a lower body size than controls (Chehab *et al.*, 1997). In addition, Yu *et al.* (1997) showed that leptin directly stimulated FSH and LH release from the murine pituitary gland and GnRH release by hypothalamic explants *in vitro*.

Furthermore, intraventricular administration of leptin to ovariectomised and oestrogen-primed mice, resulted in increased circulating levels of LH (Yu *et al.*, 1997). Finally, direct effects of leptin on ovarian function have been recently proposed by Zachow and Magoffin (1997). In their study, leptin inhibited IGF-I stimulated oestradiol production by rat granulosa cells *in vitro*.

In conclusion, research indicates that leptin might act as the signal informing the central nervous system about the state of body energy reserves, thereby initiating and maintaining reproductive cyclicity. In addition, leptin directly affects the hypothalamus, pituitary gland and ovary, and hence potentially can affect reproductive events directly.

1.2 FOLLICULOGENESIS

Folliculogenesis in domestic animals is a process which is not yet fully understood, particularly with regard to the selection of those primordial follicles which leave the pool of resting primordial follicles to begin their development. Follicle development thereafter is a continuous process until the follicle either matures and ovulates or regresses by atresia.

The ovary of domestic mammals is one of the few organs in which the number of active elements has been fixed before birth. The number of follicles present in the ovary is established during embryonic development, and the number of oocytes decreases constantly throughout the life of the female. Hirshfield (1991) has described the main events in the histogenesis of the ovary. During early embryonic development, germinal cells are localised in the amniotic sac. These cells are motile and invasive and migrate through the dorsal mesenterium to establish themselves in the primitive gonad. From this undifferentiated gonad, all the cellular components that will constitute the follicle will develop. Once the gonad has been invaded by the germinal cells, these cells lose their invasive characteristics and, together with cells of somatic lineage, enter into a phase of rapid mitotic proliferation. At the end of this proliferative phase, the somatic cells surround the germinal cells. When the germinal cells have been surrounded by the somatic cells, the former are transformed into oocytes, mitotic division ceases and they enter the process of meiotic division. The somatic cells organised around the oocytes continue to multiply during the rest of embryonic development, and they gradually engulf the oocyte, forming the primordial follicle, consisting of the oocyte arrested in the first meiotic division, surrounded by flattened pregranulosa cells, that in turn are surrounded by a basal membrane.

The primordial follicles then remain quiescent, and only a few follicles are selected to develop at a given time. The events between the reinitiation of growth of a primordial follicle until its development into a preovulatory follicle have been investigated mainly in laboratory animals. Developmental schemes for these species have been used here as a basis for the understanding of this phenomenon in cattle.

In the rat, the primordial follicle sectioned at its greatest diameter has 4 layers of pre-granulosa cells surrounding the oocyte. Hirshfield *et al.* (1987) called these pre-granulosa cells “cells of the first generation”, defining a ‘generation’ as the length of time required for the number of granulosa cells present in a follicle to double. When the cells duplicate, giving 8 cells around the oocyte, they are termed “cells of second generation”, and successively until the tenth generation which constitutes the preovulatory follicle. Microscopically, the first three generations of development are very difficult to differentiate from inactive primordial follicles, due to their slow growth rate. The follicles form the *zona pellucida*, but still lack theca interna cells. Between the fourth and seventh generation, the follicles develop theca interna. Antral spaces begin to form. The cells of the theca and granulosa layers acquire LH and FSH receptors respectively at this phase. Atresia is rarely seen during these earlier stages of development (Hirshfield, 1991).

The follicles of eighth and ninth generation represent the critical phase of development, where a follicle may develop into a preovulatory follicle or undergo atresia. In these two generations of development, follicles undergo extensive atresia. The replication capability of the granulosa cells is reduced and the antrum is fully developed. In the eighth or ninth generations of development, the follicle possesses receptors for FSH, LH, oestrogens and androgens, but the granulosa cells are still devoid of LH receptors.

Follicles of the tenth generation are characterised by developing LH receptors in the granulosa cell layer, and the theca layer differentiates into two regions, the theca interna cells, specialised in steroid production, and the theca externa layer containing actin and myosin (Hirshfield, 1991). The tenth generation follicle contains all the enzymes necessary for the production of progesterone, androgens, oestrogens and other substances (inhibin, proteoglycans, prostaglandins, plasminogen activator, etc.). Follicles of the tenth generation are capable of responding to LH or hCG administration by ovulating.

Fortune (1994) re-analysed histological data from cow ovaries obtained by Lussier *et al.*, (1987) and observed interesting similarities between Hirshfield's concept of cell generation and follicular development in the rat compared to bovine follicular development. According to her analysis, follicular development up to the preovulatory stage in cattle takes 13 generations of granulosa cell duplication. As in the rat, follicular atresia occurs in the later stages of development (10-12 generations), and only follicles of the 13th generation (>8mm in diameter) contain LH receptors in the granulosa cell layer (Xu *et al.*, 1995b).

A recent histological study of primordial follicles in cattle (van Wezel and Rodgers, 1996) has determined that the oocyte of a primordial follicle is surrounded by 24 flattened and cuboidal shaped pre-granulosa cells. However, 80% of the primordial follicles have at least one cuboidal cell layer. Thus, the presence of cuboidal cells around the bovine primordial follicle does not necessarily imply growth since, if this were the case, all the follicles in the ovary would grow at one time and the cow would become devoid of follicles in a matter of months (van Wezel and Rodgers, 1996). They also calculated that approximately 21 divisions of granulosa cells were required for a follicle to become a preovulatory follicle.

It is clear that the follicle is dependent on gonadotrophins to achieve complete development (Dufour *et al.*, 1979; Driancourt *et al.*, 1987; McNeilly *et al.*, 1991; Gong *et al.*, 1995, 1996a), and although folliculogenesis is a continual process, it could be divided into two separate phases by its gonadotrophin dependence. The first phase of folliculogenesis can take place, at least in part, in the absence of gonadotrophins. The second phase is a tonic phase, and depends primarily on gonadotrophic support (McNeilly *et al.* 1991; Driancourt, 1991).

1.2.1 INITIAL STAGES OF FOLLICULOGENESIS

As early as two weeks of age, mammalian ovaries contain follicles in all stages of development, with the exception of Graffian follicles (Land, 1970; Seidel and

Niswender, 1980). It is considered that a large number of primordial follicles start their growth soon after birth, and that many degenerate at this time. The first morphological signs of follicular growth are the proliferation of granulosa cells, which change from flattened to a cuboidal morphology, and also an increase in oocyte size, which reaches its maximum development in the early stages of follicular development (Gougeon, 1996).

Follicular growth involves the following changes:

1.- During the growth of the oocyte, tritiated uridine is incorporated into nuclear and nucleolar RNA reaching a maximum which declines later as the size of the oocyte increases (Crozet *et al.*, 1986; Crozet, 1989; Fair *et al.*, 1995). In addition, the permeability of the oocyte nucleus to precursors differs according to its size. Also endogenous RNA reserves and variation in the degree of RNA synthesis changes with time (Mariana, 1980). Thus, as the follicle grows oocyte growth declines and ceases.

2.- Follicular size increases due to cellular multiplication and the arrangement of the cells that surround the oocyte.

3.- The formation of the antral spaces among granulosa cells, which coalesce to form a single antrum.

Although the precise mechanism by which certain follicles are selected to leave the pool of resting primordial follicles and grow remains largely unknown, a number of theories have been postulated:

1.2.1.1.1 The role of gonadotrophins

Gonadotrophins control follicular growth and differentiation in four possible ways (Mariana, 1980): 1. Modifying cellular replication; 2: altering oocyte growth; 3:

inducing antrum development and 4: promoting primordial follicles to leave the pool of resting follicles.

The effect of gonadotrophins on the re-initiation of growth of primordial follicles was demonstrated by Wolff-Exalto (1982) by transplanting ovaries from new-born rats into either ovariectomised and hypophysectomised (gonadotrophin depleted) or only ovariectomised (high gonadotrophin concentrations) adult rats. After fifteen days, the ovaries transplanted to ovariectomised-only rats had more follicles containing 8-20 granulosa cells than ovaries transplanted to ovariectomised and hypophysectomised rats.

Alternatively, depletion of gonadotrophins by hypophysectomy produced a decline in the number of growing follicles at all stages of development, as early as 4 days after the hypophysectomy in mice (Wang and Greenwald, 1993a), accompanied by the disappearance of FSH and LH receptors from granulosa cells, and by loss of co-ordination between oocyte growth and granulosa cell multiplication (Wang and Greenwald, 1993a). Moreover, after hypophysectomy follicle development was inhibited and antrum formation arrested (Hirshfield, 1991; Wang and Greenwald, 1993a). However, gonadotrophin replacement restored the number of growing follicles, induced FSH and LH receptors, and synchronised granulosa cell growth with the growth of the oocyte (Wang and Greenwald, 1993b).

In sheep, hypophysectomy produced a decline in the number of healthy follicles larger than 2 mm after two days (Dufour *et al.*, 1979; Driancourt *et al.*, 1987). However, the number of follicles of up to 2 mm in diameter was not affected 8 days after hypophysectomy (Dufour *et al.*, 1979). In contrast, 70 days after hypophysectomy, ewes showed a decline in the number of preantral follicles in the ovary (Dufour *et al.*, 1979). Evidence obtained by Moore and Lintern-Moore (1979) that gonadotrophins (PMSG) increased RNA polymerase activity in the oocyte of primordial follicles of 28 day old rats compared to the control group, indicated the re-initiation of oocyte maturation. These results suggest that gonadotrophin priming might be needed for the initiation of growth of primordial follicles. Contrary to these

results, mRNA coding for FSH receptor, was localised in granulosa cells of sheep preantral follicles only after they had begun their growth and contained two layers of granulosa cells (Tisdall *et al.*, 1995).

It is possible that gonadotrophins secreted during the formation of the gonad affect adult follicular development. Gonadotrophins induce the multiplication and organisation of the cells from the *rete ovarii* and consequently increase the number of follicles at higher levels of development (Merchant, 1991).

Gonadotrophins induce antrum formation, and the antrum appears earlier and its development is greater in animals treated with gonadotrophins than in untreated animals (Mariana 1980). Gonadotrophins also induce increased growth rate and protect follicles from early atresia (Mariana, 1980; Wang and Greenwald, 1993b).

1.2.1.1.2 The role of the oocyte

Another theory proposed to explain the reinitiation of follicular growth was put forward by Mulheron *et al.* (1987) who maintained that the oocyte determines when a follicle begins its development. They localised LH and FSH in the oocytes of primordial follicles and proposed that, although gonadotrophins are necessary for follicle development, the oocyte determines when the follicle exits the latent stage. They proposed the following hypothesis. During the oestrous cycle, some follicles become a target of gonadotrophins, and FSH and LH are translocated to the nuclei of the oocytes of such follicles. In the transition period from a primordial to a primary follicle, the gonadotrophins leave the nucleus, but remain present in the cytoplasm. This capacity of the oocyte to retain gonadotrophins could explain the development of follicles up to the preantral stage in the absence of continual concentrations of gonadotrophins.

The role of the oocyte in controlling the initiation of follicular growth has been strongly supported recently. Dong *et al.* (1996) demonstrated a specific oocyte product, growth differentiating factor-9 (GDF-9). Using targetted deletion of GDF-9,

they showed that follicles only developed one layer of granulosa cells and the theca failed to differentiate in the absence of GDF-9, and this effect was independent of FSH or LH. Immunohistochemical studies have also demonstrated the presence of basic fibroblast growth factor (bFGF) in the oocytes of primordial and primary follicles (van Wezel and Rodgers, 1995). Therefore, the control of the initiation of follicular growth could well reside in the oocyte. More research will be needed to determine the role of the oocyte in the control of follicular development.

1.2.1.1.3 The role of the theca cell layer

Hirshfield *et al.* (1987) proposed that precursor theca cells are responsible for the reinitiation of follicular growth. Theca cells are not distinguishable in follicles until they are clearly in the growing phase. At first, follicles are surrounded by a layer of fibroblast-like cells generally considered to be immature theca cells. When the number of precursor theca cells around the follicle reaches a critical number, they signal the granulosa cells to begin their proliferation. This theory is supported by observations that LH administration to hypophysectomised mice increased the number of developing preantral follicles (Wang and Greenwald, 1993b). In addition, theca cells produce EGF/TGF α (Ojeda and Dissen, 1994) and androgens (Hillier *et al.*, 1994) that stimulate proliferation of theca and granulosa cells in the mouse and sheep (Ojeda and Dissen, 1994; Campbell *et al.*, 1996a).

Likewise, granulosa cells from preantral follicles secrete a theca-differentiating factor(s) that stimulates the expression of mRNA for steroidogenic enzymes and LH receptors in the theca cells (Magarelli *et al.*, 1996). Later in follicular development, granulosa cells secrete inhibin which synergises with LH to increase androgen secretion by theca cells (which is aromatised by granulosa cells) and also increase granulosa cell responsiveness to FSH (Hillier *et al.*, 1994).

1.2.1.1.4 Compartmentalisation theory

Merchant (1991) suggested that the location of the follicle within the ovary may influence the probability of a follicle to develop. The characteristics of the cellular cycle in somatic cells differ according to their position in the embryonic gonad, and possibly the same characteristic in the cell cycle applies to the cells of the *rete ovarii*. This compartmentalisation persists until the formation of the follicle. Recent evidence of this theory is the preferential growth of primordial follicles located in the medullary region of neonatal mouse ovaries in culture (Eppig and O'Brien, 1996).

In the rat, the compartmentalisation of the pool of primordial follicles is based on the replication capability of the follicular cells. This capability increases in direct relation with the size of the oocyte and the number of cells that surround the oocyte. Therefore, the probability of a follicle beginning its development is in direct relation to the number of follicular cells and size of the oocyte, and with the ability of the oocyte to synthesise nucleolar RNA (Mariana, 1980). This probability has been calculated to be close to 1 for follicles that have 40 cells or more surrounding the oocyte at the time when ovarian histogenesis is arrested. Furthermore, the size of the oocyte and its synthetic capability depends on the follicular cells that surround it. These cells establish junctions with the oocyte and transfer to it proteins and metabolites needed for oocyte growth and synthetic function (McLaren, 1980; Buccione *et al.*, 1990; Eppig, 1991).

From the primordial stage up to the antral stage follicle growth is exposed to cyclical hormonal concentrations, with periodic gonadotrophin stimuli that could either modify the multiplicative capability of granulosa cells or rescue the follicles from atresia. Although early follicular development can progress without continual gonadotrophin support, the complete absence of gonadotrophins impairs the later stages of folliculogenesis.

1.2.1.2 GONADOTROPHIN DEPENDANT FOLLICULOGENESIS

During the second phase of follicular development, the follicles become highly dependent on gonadotrophin support (McNeilly *et al.* 1991), in particular FSH at the beginning, and there is a complicated interaction between endocrine, autocrine and paracrine factors that control follicle selection and dominance (Baird *et al.*, 1991; Driancourt *et al.*, 1987; Fortune *et al.*, 1991; Webb *et al.*, 1994; Campbell *et al.*, 1995). This phase of follicular development consists of three processes defined as Recruitment, Selection and Dominance.

1.2.1.2.1 Recruitment.

Recruitment consists of the gonadotrophin-stimulated growth of a group of follicles (follicular wave), from which a single follicle will become dominant and develop to preovulatory size. For a follicle to be recruited, it must have reached the gonadotrophin- dependent phase, which in the bovine is reached when the follicle is around 4-5 mm in diameter (Gong *et al.*, 1995; 1996a). In the immediate period after ovulation, there is an increase in FSH concentration, and this post-ovulatory FSH rise induces the recruitment of follicles for the next oestrous cycle (Turzillo and Fortune, 1990). In cattle, between 5 to 6 follicles are recruited in each follicular wave, and recruitment is preceded by a rise in FSH concentrations (Adams *et al.*, 1993; 1994; Sunderland *et al.*, 1994). FSH is indispensable to sustain the growth of antral follicles, and if FSH support is withdrawn (*e.g.* by hypophysectomy) atresia of all antral follicles results (Driancourt *et al.*, 1987; Dufour *et al.*, 1979; Wang and Greenwald, 1993b).

In cattle, studies on hypophysectomised animals are lacking. However, gonadotrophin depletion (Gong *et al.*, 1996a) of the pituitary achieved by chronic treatment with GnRH agonist (Buserelin) showed that follicles are dependent on FSH to grow beyond 4 mm in diameter and up to 7-9 mm at which stage they transferred their gonadotrophin dependence to LH pulses. Therefore, gonadotrophins appear to be the primary drive for follicle growth once they reach the gonadotrophic-dependent

stage. The follicle size reached at this stage is species-specific (mouse: preantral stage, Hirshfield, 1991; ovine: >2mm, Dufour *et al.*, 1979; McNeilly *et al.*, 1991; bovine: >4mm Gong *et al.*, 1995; 1996a).

1.2.1.2.2 *Selection and Dominance.*

Once a group of follicles has been recruited, selection of the dominant follicle takes place, in part by interference by the largest follicle with the gonadotrophin supply to smaller follicles of that cohort. Two possible mechanisms exist:

i) An indirect mechanism, where the largest (dominant) follicle inhibits the growth of the other follicles in the cohort, through the production of substances such as inhibin and oestradiol that will act on the hypophysis reducing FSH secretion to levels insufficient for the survival of subordinate follicles (Baird *et al.*, 1991; Beard *et al.*, 1989; Larson *et al.*, 1991). Both oestradiol and inhibin, when administered to sheep or cattle, are able to suppress FSH release (Baird *et al.*, 1991; Gong *et al.*, 1997), whilst passive or active immunoneutralisation of oestradiol or inhibin results in increased FSH concentrations (Baird *et al.*, 1991).

ii) A direct mechanism, where the dominant follicle inhibits the growth of the cohort through the secretion of paracrine and endocrine factors that reduce the sensitivity of the follicles to FSH. Although the nature of the substance(s) responsible for this direct inhibition of follicular growth is unknown, extensive research is being carried out in this area. In cattle, administration of inhibin-free follicular fluid after ovulation resulted in a delay in the development of the follicular wave for 2 days (Turzillo and Fortune, 1990). In contrast, if FSH was given before the postovulatory peak of FSH, the first wave of follicular development was advanced. Similarly, a decrease in pituitary FSH secretion with a subsequent effect on follicular dynamics has been found in postpartum animals (Hinshelwood *et al.*, 1991).

Furthermore, an atretogenic factor might exist in follicular fluid, as administration of oestradiol- and inhibin-free follicular fluid decreases follicular development in sheep

(Campbell *et al.*, 1991) and delays the return to oestrus after luteolysis in cattle (Law *et al.*, 1992). Moreover, this putative factor inhibited oestradiol production and the proliferation of ovine and bovine granulosa cells *in vitro* (Baxter *et al.*, 1995).

Dominance refers to the selective atresia provoked in subordinate follicles by the growth of a large preovulatory follicle (see above). How the selected dominant follicle differs from other follicles in the cohort is unknown. It is possible that the dominant follicle reaches a stage of differentiation at which it can sustain growth in the presence of lower circulating concentrations of FSH (Fortune, 1994). The development of LH receptor in the granulosa cells will give the follicle an advantage when FSH concentrations are low (Driancourt, 1991). This presumption is supported by the appearance of LH receptors on the granulosa cells when the bovine follicle reaches 8-9 mm in diameter (Xu *et al.*, 1995b), and the observation that follicles need LH to grow beyond 8-9 mm in diameter (Gong *et al.*, 1995). Furthermore, increased LH pulsatility caused by subluteal progesterone concentrations maintained the growth of the dominant follicle (Stock and Fortune, 1993; Savio *et al.*, 1993), highlighting the trophic effect of LH on the dominant follicle. Although it is clear that LH plays a principal role in the function of the dominant follicle, basal concentrations of FSH are still needed by the dominant follicle as FSH withdrawal causes atresia of the dominant follicle (Fortune, 1994; Gong *et al.*, 1995; 1996a).

The dominant follicle in cattle is capable of ovulating in response to LH/hCG (Price and Webb, 1989; Schmitt *et al.*, 1996b). However, although the dominant follicle has this capability it will not ovulate during the oestrous cycle unless there is a decline in progesterone concentrations (Kesner *et al.*, 1981) and the oestradiol produced by the follicle induces a preovulatory LH peak (Baird and McNeilly, 1981; Hansel and Convey, 1983; Stumpf *et al.*, 1989). In the preovulatory stage, the maturation of the thecal compartment of the preovulatory follicle, in response to increased pulsatile concentration of LH, results in enhanced androstenedione production and expression of mRNA for P450 side-chain-cleavage (P450scc) and P450 17 α -hydroxylase in the theca cells. These changes correspond with increased expression of P450scc and 3 β -

hydroxysteroid dehydrogenase (3 β HSD) in the granulosa cells (Bao *et al.*, 1997), without alteration in the expression of P450 aromatase (Tian *et al.*, 1995).

1.2.1.3 FOLLICULAR ATRESIA

With the exception of the dominant ovulatory follicle, atretic degeneration is the fate of at least 99 % of the follicles present in the ovary. Attrition of non-selected follicles may be an evolutionary mechanism to ensure that only follicles containing the most healthy oocytes progress to ovulation (Hsueh *et al.*, 1994), and ensuring the correct number of ovulations for the species (Rajakoski, 1960; Campbell *et al.*, 1995). In the mammalian ovary, atresia occurs at several stages of development. During the development of the primitive gonad there is degeneration of primordial germ cells coinciding with the onset of meiosis (Hirshfield *et al.*, 1991; Billig *et al.*, 1996). After completion of the formation of the gonad, the follicles whose oocytes (cells dividing by meiosis) are not completely surrounded by pre-granulosa cells (50-70% of the original number of oocytes) will also degenerate (Hirshfield *et al.*, 1991; Tilly, 1993). Quiescent follicles will remain in the ovary until the re-initiation of growth, after which atresia can occur at any stage, but is more frequently observed at the preantral, penultimate stages of development (selectable follicles) and degeneration of the potentially ovulatory follicle can occur (Hsueh *et al.*, 1994; Billig *et al.*, 1996). As stated above, gonadotrophins play a primary role in the maintenance of follicular growth at all stages of development from the early antral stage (Chun *et al.*, 1996), through to the preovulatory stage.

Hirshfield (1991) suggested that atresia in developing antral follicles occurs at the time when the granulosa cells are in the phase of greatest mitotic replication. Thus their need for oxygen and nutrients is at its maximum. This situation, together with the enlargement of the follicular wall, may result in follicular oxygen deficiency. Therefore, it is possible that FSH enables the granulosa cells to survive in hypoxic conditions (Hirshfield, 1991). FSH declines when a follicle achieves dominance and levels remain low until the follicle is ovulated or its dominance ceases. However,

despite lower circulating FSH, the amount of FSH reaching the dominant follicle may be unchanged or even increased since the dominant follicle is much more highly vascularised (Gougeon, 1996). Once the follicle has passed this hypoxic phase, the dominant bovine follicle does not require high levels of FSH as it switches its gonadotrophin dependence to LH (Gong *et al.*, 1996). Moreover, the production of autocrine or paracrine factors within the ovary may enable the preovulatory follicle to overcome the adverse conditions that arise after the decline of FSH support (Gougeon, 1996; Lobb and Dorrington, 1992; Adashi, 1992b; Redmar *et al.*, 1996).

Histologically, atretic follicles are characterised by increased pyknotic nuclei in the granulosa cells, loose granulosa cells adjacent to the follicular cavity and (in later degenerative stages) disruption of the basal lamina and a reduction of granulosa cell number (Ireland and Roche, 1983; Takagi *et al.*, 1993; Grimes *et al.*, 1987; Yoshinaga-Hirabayashi and Osawa, 1994; Logothetopoulos *et al.*, 1995). DNA synthesis also declines in atretic granulosa cells (Hirshfield, 1989).

1.2.1.3.1 Follicular atresia and steroid production

Steroid measurements in antral follicles showed that follicular fluid oestradiol content (England *et al.*, 1981; Staigmiller *et al.*, 1982; Spicer *et al.*, 1988), and aromatase activity, as indicated by the oestradiol to androstenedione ratio (Carson *et al.*, 1981), increase together with follicle diameter. However, during follicular atresia, there are changes in the pattern of steroid production, reflected in reduced ratios of oestradiol to androgens (Carson *et al.*, 1981) or oestradiol to progesterone (Grimes *et al.*, 1987; Jolly *et al.*, 1994) in the follicular fluid. Furthermore, the ratio of oestradiol to androstenedione or progesterone in follicular fluid has been used as a means of classifying follicular health (Spicer *et al.*, 1988; Sunderland *et al.*, 1994; De La Sota *et al.*, 1996). These changes in steroid concentrations in the follicular fluid of healthy and atretic follicles have recently been shown to match changes in expression of proteins and mRNA for steroidogenic enzymes. Granulosa cell expression of P450 aromatase, the enzyme responsible for converting androgens to oestrogens (Hillier *et*

al., 1994) decreased as atresia advanced (Garrett and Guthrie, 1996; Xu *et al.*, 1995a; Yoshinaga-Hirabayashi and Osawa, 1994). In addition, expression of 17 α -hydroxylase/C17-C20 lyase, the enzyme responsible for converting progesterone to androstenedione in the theca cell layer, decreased as atresia progressed (Garrett and Guthrie, 1996; Yoshinaga-Hirabayashi and Osawa, 1994).

1.2.1.3.2 Apoptosis

Cell demise within the follicle occurs by apoptotic death of granulosa cells (Hughes and Gorospe, 1991; Tilly, 1993; Hsueh *et al.*, 1994; Jolly *et al.*, 1994; Chun *et al.*, 1996; Hakuno *et al.*, 1996; Tilly, 1996) and theca cells (O'Shea *et al.*, 1978). Apoptosis is a genetically controlled process of physiological cell death which regulates tissue and organ development (Falcieri *et al.*, 1994). Apoptosis is most commonly seen in cells derived from highly mitotic lines (Zakeri *et al.*, 1995), and some authors consider apoptosis to be an integral part of the cell cycle (Meikrantz and Schlegel, 1995). As with many other tissues, apoptosis occurs in healthy and atretic follicles (Jolly *et al.*, 1994) and appears to be the preferential mechanism in regulating cell death in physiologically regressing tissues. Thus, follicular atresia is a controlled process (Zelesnik *et al.*, 1989; Hughes and Gorospe, 1991; Hsueh *et al.*, 1994; Jolly *et al.*, 1994; Tilly, 1996).

The term "apoptosis" has been used synonymously with "programmed cell death". However, apoptosis is rather a type of cell death characterised by certain morphological and biochemical features, and although programmed cell death can occur by apoptosis, one does not imply the other (for review see Majno and Joris, 1995; Duke *et al.*, 1996). Apoptosis was first described by Kerr *et al.* (1972) from morphological observations, and these are still used as a standard for its definition. Ultrastructurally, apoptosis takes place in two stages (Kerr *et al.*, 1972; Majno and Joris, 1995). The first stage is cell death and formation of apoptotic bodies, which is then followed by phagocytosis and degradation by other cells. Apoptotic cells show characteristic changes. The nucleus is particularly affected, suffering early collapse

and chromatin condensation. The cell membrane 'blebs', nuclear fragmentation subsequently appears, and at very late stages there is cytoplasmic condensation with preservation of the plasma membrane. Cell fragments are phagocytosed by neighbouring cells and infiltrating macrophages without inducing an inflammatory response (Kerr *et al.*, 1972; Kerr *et al.*, 1994; Falcieri *et al.*, 1994; Zakeri *et al.*; 1995). In the second stage, apoptotic bodies suffer autolytic changes similar to those observed in necrosis. However, they are still recognisable as apoptotic bodies by the characteristic presence of condensed chromatin (Kerr *et al.*, 1972; Majno and Joris, 1995). Apoptosis is a very fast process and can be accomplished in minutes (Majno and Joris, 1995)

The factors that implement cell death are already present in the cytoplasm and nucleus of the cell and do not require *de novo* synthesis for activation (Vaux and Strasser, 1996). Apoptosis comprises a series of phases that include an external signal and receptor and signal transduction pathways. These finally converge in a common activation of calcium-dependent endonucleases (Wyllie, 1980; Falcieri *et al.*, 1994; Vaux and Strasser, 1996) resulting in DNA cleavage into nucleosomal or oligo-nucleosomal fragments (multiples of 185 base pairs) that can be visualised as a ladder pattern of DNA bands after gel electrophoresis.

The events and effectors of apoptosis are not all known, however rapid advances are being made and a clear picture is beginning to arise. The components of the apoptotic process vary depending on the cell type and stage of differentiation (Martin *et al.*, 1994; Schwarzman and Cidlowski, 1993; Vaux and Strasser, 1996). The signal that initiates apoptosis could be a factor acting on the cell surface, such as an atretogenic factor or withdrawal of a trophic factor (Schwarzman and Cidlowski, 1993; Wertz and Hanley, 1996). In the follicle, the initiation signal could be the withdrawal of gonadotrophins (Tilly, 1996) or growth factors like IGF-I (Hsueh *et al.*, 1994), perhaps blocked by IGF-BPs. Tumor necrosis factor (TNF), acting through its receptor, is capable of inducing apoptosis in follicular cells (Kaipia *et al.*, 1996). In addition, Fas/Apo-1/CD95, a cell surface protein found in activated T-cells and similar to the TNF membrane receptors (Hsueh *et al.*, 1994; Kitson *et al.*, 1996), has

been found in the granulosa cells of atretic, but not healthy follicles (Hakuno *et al.*, 1996).

Regardless of what initiates apoptosis, the downstream mechanism appears to involve the same effectors in all the cells (Martin *et al.*, 1994; Ueda and Shah, 1994) and ovarian cells are no exception (Billig *et al.*, 1996; Tilly, 1996). There are several gene products known to regulate the apoptotic process, though more are expected to emerge. The apoptotic effectors are cysteine proteases belonging to the ICE (interleukin-1 β -converting enzyme) gene family and Ca²/Mg²-dependent endonucleases (Schwarzman and Cidlowski, 1993; Tilly, 1996; Vaux and Strasser, 1996). These enzymes are responsible for the disruption of the cellular scaffolding and cleavage of DNA in the internucleosomal spaces. Other gene products regulate the action of the former enzymes. Of them, the most prominent is Bcl-2. Bcl-2 encodes for a protein that is bound to mitochondrial membranes and inhibits apoptosis triggered by a number of stimuli. Thus Bcl-2 may inhibit a central step in the apoptotic pathway (Hockenbery, 1995). Indeed, Bcl-2 concentration is higher in follicles destined to ovulate (Tilly, 1993), and Bcl-2 over-expression in transgenic mice reduced the number of follicles undergoing apoptosis and increased litter sizes (Hsu *et al.*, 1996). Other members of this gene family are known to either enhance (BAX, Bcl-X_{short}, BAD) or repress (Bcl-X_{long}) apoptosis (Billig *et al.*, 1996; Tilly, 1996; Martin *et al.*, 1994; Ueda and Shah, 1994). Finally, p53 protein is present in the nucleus of the cells. This protein stops the cell cycle and triggers repair processes to the DNA when it gets damaged. However if DNA repair fails, p53 accumulation triggers apoptosis (Ueda and Shah, 1994). P53 has been observed in nuclei of cells from follicles destined for atresia (Tilly, 1996).

1.2.1.3.3 Intra-ovarian control of follicular development

The control of follicular development is dependent upon the interaction of systemic and intraovarian regulatory mechanisms. A key component of these intraovarian mechanisms is the IGF system. As described above, IGFs are present in follicular fluid and potently stimulate the proliferation and steroidogenesis of follicular cells *in*

vitro. In this section evidence for the IGF system as an intraovarian controller of follicular development is reviewed.

IGFs. A number of studies have reported the secretion of IGF-I and/or -II by bovine, porcine and murine granulosa cells. However, in most of these early studies, IGFs were measured by radioimmunoassay after the extraction of IGFBP by methods other than acid-gel chromatography, with the possibility that IGFBP removal was incomplete. From data obtained by such methods (Spicer *et al.*, 1993b) and by comparison with evidence gathered in other species, it was assumed that IGFs are produced within the bovine follicle (Spicer and Echterkamp, 1995).

Nevertheless, when reviewing the literature that reported the expression of IGF-I and/or -II mRNA (Table 1.1) it becomes apparent that IGF mRNA expression is tissue- and species-specific. While in rodents IGF-I mRNA expression is localised to the granulosa cells of healthy follicles, there is no expression of IGF-II mRNA by the follicle (Adashi *et al.*, 1991; Botero *et al.*, 1993; Oliver *et al.*, 1989; Hernandez, 1995). In the pig IGF-I and -II mRNA expression was first localised in the follicular wall (Samaras *et al.*, 1993) and whole ovarian homogenates (Samaras *et al.*, 1994). Recently, compartmentalised expression has been described. IGF-I mRNA is expressed in granulosa cells of healthy follicles and IGF-II mRNA is expressed in both the theca and granulosa compartments (Samaras *et al.*, 1996; Zhou *et al.*, 1996). In contrast, in the human ovary IGF-II is expressed in granulosa cells whereas IGF-I is not expressed in mature follicles (El-Roeiy *et al.*, 1993; Zhou and Bondy, 1993; Voutilainen *et al.*, 1996), but IGF-I mRNA is expressed in theca cells of small antral follicles (El-Roeiy *et al.*, 1993).

In ruminants, there is controversy regarding the expression of IGF mRNAs within the follicle. A recent report, using *in situ* hybridisation, suggested that ovine granulosa and theca cells express IGF-I mRNA (Leeuwenberg *et al.*, 1995). However, using similar techniques Perks *et al.* (1995) were unable to detect IGF-I mRNA from either cell type. In cattle, IGF-I mRNA was found in granulosa cells of preovulatory follicles after FSH superovulatory stimulation (Spicer *et al.*, 1993b).

However, a certain degree of luteinisation may have occurred in these granulosa cells.

IGFBPs. IGF-binding proteins in follicular fluid vary according to the developmental status of the follicle. In ovine follicles, the IGF-binding activity of growing follicles was relatively low compared with a higher IGF-binding activity of atretic follicles (Monget *et al.*, 1993). Furthermore, besides changes in the total amount of IGFBP activity, the proportional amount of the different IGFBPs also changes with follicular development. IGFBP-2, -3, -4, and -5 were found in follicles <5mm in diameter (De La Sota *et al.*, 1996; Funston *et al.*, 1996). Follicle growth was accompanied by a decrease in IGFBPs of small molecular mass (IGFBP-2, -4 and -5) while IGFBP-3 was unchanged (De La Sota *et al.*, 1996; Funston *et al.*, 1996; Stewart *et al.*, 1996). When the follicle reached dominance, it contained significant amounts of IGFBP-3 and low amounts of other IGFBPs (Funston *et al.*, 1996; Stewart *et al.*, 1996). However, the amounts of smaller molecular weight IGFBPs increased in late dominant follicles (Stewart *et al.*, 1996) or with atresia (De La Sota *et al.*, 1996). These changes in quantity and quality of IGFBPs within the follicle may control the local availability of systemic or locally-produced IGFs.

Table 1.1. Reported expression of IGF-I and/or IGF-II mRNA in granulosa and theca cells in different species.

Species	Reference	Granulosa	Theca
Rat	Botero <i>et al.</i> , 1993	IGF-I	
	Hernandez, 1995	IGF-I	IGF-II
	Oliver <i>et al.</i> , 1989	IGF-I	
	Adashi <i>et al.</i> , 1991	IGF-I	
Pigs	Samaras <i>et al.</i> , 1993	IGF-I and-II (follicle shells)	
	Samaras <i>et al.</i> , 1994	IGF-I and-II (whole ovarian homogenates)	
	Samaras <i>et al.</i> , 1996	IGF-I	IGF-I (scraped follicular wall)
	Zhou <i>et al.</i> , 1996	IGF-I and IGF-II	IGF-II
	Yuan <i>et al.</i> , 1996	IGF-I	IGF-II
Humans	Adashi <i>et al.</i> , 1991	IGF-II	
	El-Roeiy <i>et al.</i> , 1993	IGF-II	IGF-I and IGF-II
	Zhou and Bondy, 1993	IGF-II	
	Voutilainen <i>et al.</i> , 1996	IGF-II	IGF-I and IGF-II
Sheep	Leeuwenberg <i>et al.</i> , 1995	IGF-I	IGF-I
	Perks <i>et al.</i> , 1995		IGF-II
Cattle	Spicer <i>et al.</i> , 1993b	IGF-I (superovulated cattle)	

AIMS

The aims of this work were:

To study the relationship between nutrition, metabolic hormones (growth hormone, insulin, and insulin-like growth factor) and follicular development in cattle.

To develop a model for the study of the direct action of endocrine factors on bovine granulosa cells.

To examine the putative role of IGF-I as an intraovarian regulator of follicular development.

2. MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

All experimental animal procedures were carried out in compliance with the “Animal (Scientific Procedures) Act, 1986”.

Animal experiments were carried out at the Institute’s Large Animal Unit (Dryden, Mountmarle) or at the Institute’s farm (Blythbank). All animals used were sexually mature, and were adapted before hand to the environmental conditions, management and diets. Animals were kept together in a large covered pen or at pasture, under natural photoperiod. Body weight was monitored once a week.

2.2 OESTRUS SYNCHRONISATION

The desired stage of the oestrous cycle was achieved by synchronising oestrus by the insertion of progestagen-releasing intravaginal devices (PRID; Sanofi Animal Health, P.O. Box 209, Rhodes Way, Watford, Herts, U.K.) for 12 days, followed by an intramuscular injection of 500 µg of prostaglandin F2α analogue (Estrumate, Coopers Animal Health Ltd. Crewe, Cheshire, UK) at the time of PRID withdrawal. Heat was detected by behavioural oestrus, aided by a KAMAR heatmount detector (Kamar Inc., Steamboat Springs, CO, USA).

2.3 BLOOD SAMPLING

Blood samples were collected by jugular venipuncture into prechilled glass tubes containing 100 µl of sodium citrate (0.35g/ml) and kept ice-cold until centrifugation. Centrifugation (1000g for 30 minutes) was carried out within 30 minutes of the first collected sample. Plasma was pipetted out, aliquoted (3-4 ml) and stored at -20 C until assayed.

Frequent blood samples were taken via indwelling jugular cannulae. Animal cannulation was performed the day before the sampling session. Cannulae were inserted into the jugular vein of standing restrained heifers through a small skin

incision. Local analgesia was with lidocaine (Lignol, Arnolds Veterinary Products Ltd., Cirencester). Cannulae were then flushed with saline containing heparin (20 U/ml), stoppered and secured in place by two stitches. The morning after, cannulae were checked for patency and flushed with saline containing heparin. Blood samples (15 ml) were withdrawn into a prechilled tube containing sodium citrate and centrifuged for plasma harvesting. After each sample, heparinised saline was flushed through the cannula to ensure its patency throughout the sampling period. Animals were decannulated the day after the bleeding session was finished.

2.4 ULTRASOUND EXAMINATION OF THE OVARIES

Ovarian ultrasound examination was carried out using an ultrasound scanner (Aloka Echo Camera SSD-210 DX II) fitted with a 7.5 MHz linear array transrectal probe (Aloka Co. Ltd., Tokyo, Japan). The scanning procedures were as previously described by Pierson and Ginther (1988). Briefly, the rectum was evacuated manually of faeces. The probe, covered in a conductive gel, was introduced through the rectum. Following the uterus dorsally and then laterally, the ovaries were located and examined without directly manipulating them or the reproductive organs. The ovary was scanned by slowly moving the probe from the medial to the lateral side of the ovary. Ovarian structures were recorded at the time of the examination and on videotape for later analysis, using a videoplayer (Panasonic HS800) fitted with slow motion replay. The internal diameter of follicles were measured from their echogenic images and classified according to size into small (<4 mm diameter), medium-sized (4-8 mm) and large follicles (>8 mm). The presence or absence of the corpus luteum and other ovarian structures (*i.e.* ovarian cysts) were also recorded.

2.5 CELL CULTURE

2.5.1 MATERIALS

Medium 199, Hepes, amphotericin, L-glutamine, Dulbecco's phosphate-buffered saline with (DPBS⁺) and without calcium and magnesium (DPBS⁻) were purchased

from Gibco (GIBCO BRL, Life technologies Ltd., Paisley, Renfrewshire, UK.). McCoy's 5a medium with sodium bicarbonate, Dulbecco's minimum essential medium:Hams's F12 medium (1:1 DMEM:F12), penstrep (containing 10,000 IU penicillin and 10 mg Streptomycin per ml), bovine serum albumin, tissue culture grade transferrin, selenium, bovine insulin, neutral red, trypsin, androstenedione, oestradiol, collagenase, hyaluronidase, protease (pronase E from *Streptomyces griseus*), deoxyribonuclease, foetal donor serum and trypan blue were purchased from Sigma (Sigma Chemical Co.Ltd. Poole, Dorset, UK.). Bovine follicle-stimulating hormone (USDA-bFSH-I-2; bioactivity potency 854 IU mg⁻¹), was generously donated by the U.S. Department of Agriculture. Ovine luteinising hormone (NIDDK-oLH-S26; bioactivity 2.3 IU/mg) was generously donated by the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases (Torrance, Ca. USA). Human recombinant IGF-I and the IGF-I analogue Long R3 IGF-I (LR3-IGF-I which does not bind to IGF-binding proteins), were purchased from Gropep (Gropep Pty Ltd. Adelaide, S.A. Australia).

2.5.2 GRANULOSA CELL ISOLATION

Ovaries were collected at the local abattoir at random stages of the oestrous cycle and placed immediately into collection medium (Medium 199, with 20 mM Hepes and 100 IU/ml of penicillin, 0.1 mg/ml streptomycin and 1 µg/ml amphotericin) at 37°C, and transported to the laboratory. Ovaries were trimmed and placed in 70% ethanol for 30 seconds, then rinsed and maintained in collection medium (37°C) until dissection. The follicles were blunt-dissected and classified according to external diameter into small (<4 mm diameter), medium-sized (4-8 mm) and large (>8 mm), eliminating follicles with opaque or bloody follicular fluid. Follicle size categories were selected based on their gonadotrophin dependence and changes in the expression of steroidogenic enzymes and LH receptor (Xu *et al.*, 1995a,b). From around 8mm in diameter onwards, follicles express LH receptors on the granulosa cells (Xu *et al.*, 1995b), and require pulsatile LH stimulation to continue growing (Gong *et al.*, 1996a). At 4mm there is an increase in expression of P450-aromatase (Xu *et al.*, 1995a) and their growth will be halted if FSH is suppressed (Gong *et al.*,

1995; 1996a). After follicle dissection, follicular fluid was aspirated from medium-sized and large follicles, using a 1 ml syringe fitted with a 23 gauge needle (this step was not necessary for the small follicles), and then follicles were hemisected in a petri dish containing 5 ml of DPBS⁻. For each follicle size category, granulosa cells were removed by gently scraping the follicle wall with an inoculation loop and pooled. The resulting granulosa cell suspension was transferred into a 15 ml centrifuge tube where it was supplemented with 10 ml of culture medium and centrifuged at 800 X g for 10 minutes. After a second wash, the cells were resuspended in 5 ml of culture medium and cell number and viability estimated in a haemocytometer using Trypan blue exclusion.

2.5.3 GRANULOSA CELL CULTURE

Granulosa cells were cultured in 96 well plates (Nunclon, Life technologies, Paisley, UK.), in McCoys 5a medium with sodium bicarbonate (0.22%) supplemented with 20 mM HEPES, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 3 mM L-glutamine, 0.1% BSA, 10^{-7} M androstenedione, 2.5 µg/ml transferrin, and 4 ng/ml selenium in the complete absence of serum, except when indicated. Cells (50 µl) were seeded into each well containing 200 µl of pre-equilibrated media and incubated in a humidified atmosphere with 3.8% CO₂ at 37 °C for up to 6 days, replacing 70% of the media with fresh media every 48 hours.

2.5.4 THECA CELL ISOLATION

After isolation of the granulosa cells, the remainder of the follicle wall was dispersed by enzymatic digestion to isolate the theca cells (Campbell *et al.*; unpublished). Briefly, theca shells were digested at 37°C in an enzymatic mixture containing collagenase (5 mg/ml), protease (1 mg/ml) and hyaluronidase (1 mg/ml) in 20 ml of DPBS⁻. After 20 min, 200 µl of deoxyribonuclease (2 mg/ml) was added and incubation continued for further 20-30 min. The reaction was stopped by addition of foetal donor serum (1 ml). The solution was allowed to settle and the supernatant containing the cells was transferred to centrifuge tubes where it was diluted with theca cell culture medium and centrifuged. After a second wash, the cells were

resuspended in 15 ml of culture medium and cell number and viability estimated in a haemocytometer using Trypan blue exclusion.

2.5.5 THECA CELL CULTURE

Theca cells were cultured in DMEM:F12 media supplemented with bicarbonate, 15 mM HEPES, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 3 mM L-glutamine, 0.1% BSA, 10^{-7} M oestradiol, 2.5 µg/ml transferrin, and 4 ng/ml selenium in the complete absence of serum. The cells were incubated in a humidified atmosphere with 3.8% CO₂ at 37 °C replacing media every 48 hours.

2.5.6 PREPARATION OF SUBSTANCES TESTED

Hormone treatments were prepared before hand in culture media at 5 times the required concentration. At 48 and 96 hours of culture, treatments were prepared in sterile 96 well plates by adding 50 µl of the corresponding treatments to each well and making the volume up to 250 µl. Plates were equilibrated in the incubator for three hours before medium was changed. Spent media were stored at -20°C until hormone quantitation.

The serum treatment involved precoating the culture wells by adding 50 µl of foetal donor serum and leaving the plate overnight in the incubator. The next morning, serum was removed and the wells rinsed twice with 250 µl of culture medium.

2.5.7 DETERMINATION OF CELL NUMBER.

At the end of the culture period, media were collected and granulosa cell number was estimated by the uptake of the vital dye, neutral red, as described by Campbell *et al.* (1996a). Briefly, 200 µl of culture medium containing 10 µg/ml of neutral red was added to each well and incubated for 3h. Cells were then fixed with 200 µl of formol-calcium (4% formaldehyde with 1% calcium chloride) for 2 min. The colour was developed by removal of formol-calcium and addition of 200 µl of acetic acid-ethanol (50% ethanol and 1% acetic acid). After incubating for 15 min, the

absorbance of the solution was measured at 540 nm. The relationship between absorbance and cell number was established by incubating 3-5 million cells with the same solution of neutral red. The cells were trypsinised (0.2% trypsin and 0.08% EDTA in DPBS⁻) and counted in an haemocytometer. Cells were then centrifuged, and the pellets fixed with 1 ml of formol-calcium and acetic acid-ethanol. The resulting solution was serially diluted in acetic acid-ethanol and quadruplicate 200 μ l aliquots were placed in a 96 well plate and the absorbance determined at 540 nm. A regression equation was fitted to determine the relationship between absorbance and cell number. In every case, this was found to be linear. The method had a lower detection limit of 3.5×10^3 cells and a coefficient of variation of <2%.

2.6 PREANTRAL FOLLICLE CULTURE

2.6.1 PREANTRAL FOLLICLE ISOLATION

Bovine preantral follicles were dissected from ovaries at random stages of the oestrous cycle as described by Ralph (1996). Briefly, ovarian cortical slices (1-2 mm thick) were cut from the ovarian surface. Preantral follicles were visualised under the dissecting microscope and manually isolated using 25 G needles. Between 16 to 28 healthy follicles were obtained on each occasion.

2.6.2 FOLLICLE CULTURE AND TREATMENTS

Preantral follicles were cultured in a system developed for bovine granulosa cells (see above). Follicles were cultured individually in 96 well plates with 250 μ l of culture medium supplemented with 10 ng/ml of insulin. Follicles were incubated in a humidified atmosphere with 3.8% CO₂ at 37°C and medium was replaced every 6 days with fresh medium. Hormonal treatments were prepared in sterile 96 well plates, and equilibrated in the incubator for 3 hours before the medium was changed.

2.6.3 ASSESSMENT OF FOLLICULAR GROWTH AND HEALTH

After isolation, follicles were examined for their integrity. Only healthy follicles (with oocyte and granulosa cells completely surrounded by basement membrane and theca-stroma layer) were cultured. Follicle degeneration was marked by breakdown or rupture of the basement membrane, opacity of granulosa cells or extrusion of the oocyte from within the follicle. Follicles were fixed with 4% paraformaldehyde and stained with 5 mg/ml ethidium bromide (Sigma) for 15 min, then examined by confocal microscope (BioRad MRC-500; BioRad Laboratories Ltd., Hertfordshire, UK) to confirm the presence of an antrum. Follicle and oocyte diameter were measured using a calibrated eye piece fitted in an inverted microscope.

2.7 METABOLITE ANALYSIS

Plasma metabolite concentrations were analysed commercially (SAC, Veterinary Investigation Centre, Perth). Urea concentrations were measured by a urease/glutamate dehydrogenase method (Technicon system, Miles Inc. Diagnostics, Tarrytown, NY), and glucose concentrations were measured by the hexokinase method (Technicon). The coefficient of variations were 1.8% and 1.6% and the detection limits were 0.0081 and 0.045 absorbance units (A) per mmol/L respectively. 3 β -hydroxybutyrate (BOHB) concentrations were measured using 3-hydroxybutyrate-dehydrogenase (Ranbut, Randox Laboratories Ltd. Ardmore, Co. Antrim, UK). The coefficient of variation was 4.3%, and the detection limit 0.049 A per mmol/L.

2.8 RADIOIMMUNOASSAYS (RIA)

Hormone concentrations were measured in plasma and spent culture media samples by specific double antibody RIAs. Stock standards were kept frozen at -40°C and diluted in assay buffer to the desired concentration, to give a wide range of standards. Unknown samples were diluted in assay buffer. Specific primary antibody (raised in rabbits, guinea pigs or sheep) was added to all tubes, except non-specific binding

tubes, and incubated at 4°C for the predetermined length of time (see Appendix). Iodinated label was added ($14\text{--}16 \times 10^3$ cpm in 100 μl) together or after incubation with the first antibody and incubation continued. Bound and unbound hormone were separated by adding a specific second antibody (Scottish Antibody Production Unit, SAPU, Law Hospital, Carlisle, Lanarkshire, Scotland) raised against IgG of the species of origin of the first antibody, incorporating 10% of 0.1M diaminoethanetetra-acetic acid disodium salt (EDTA,Na_2), together with normal serum (SAPU) from the same species. Assay tubes were then incubated overnight at 4°C, 1 ml of assay buffer added and tubes spun at $1,800 \times g$ for 30 minutes at 4°C. Supernatants were tipped off and tubes allowed to drain before the radioactivity remaining in the pellet was counted in an automatic gamma counter (1277 gammacounter, Wallac, EG&G, Crownhill Business Centre, Milton Keynes).

Where possible, all samples from a particular experiment were analysed in a single RIA. FSH, insulin, growth hormone, progesterone, insulin-like growth factor-I, oestradiol and androstenedione (see Appendix) were all measured by modifications of this radioimmunoassay procedure.

2.8.1 COMPETITIVE RADIOIMMUNOASSAY FOR IGF-I

IGF-I concentrations were measured by radioimmunoassay, as described by Armstrong *et al.* (1990) and validated for the bovine (Gong *et al.*, 1991). To evaluate extraction efficiency and measure IGF-I in non-extracted culture samples, a competitive IGF-I assay was developed based on the procedure described by Blum *et al.* (1988). Samples were diluted in acid buffer to dissociate the IGFs from IGFBPs. Interfering IGFBPs were then blocked with excess IGF-II (2 ng/tube; GroPep, Australia) in neutralising buffer (0.1M sodium phosphate, 0.05M NaCl, 0.1% BSA, 0.02% NaN_3 , 0.1% Triton X-100; pH 7.8) added at the same time as the first antibody. The crossreactivity of IGF-II with the IGF-I antiserum was 0.5%. The sensitivity of the assay was 0.11 ng/ml.

2.9 IGFBP EXTRACTION PROTOCOLS

Ethanol:acetone:acetic acid (EAA) extraction was performed as described by Enright *et al.* (1989). Briefly, 100 μ l of sample was acidified with 400 μ l of EAA (60:30:10 v/v) and incubated for 30 min at room temperature. After centrifugation of the sample (1800g for 30 min), 250 μ l of the supernatant was neutralised with 100 μ l of 0.855M Tris (pH 8.5) and re-centrifuged (1800g for 30 min).

EAA plus cryoprecipitation (EAA-C) involved EAA extraction as described above, plus an additional step where samples were incubated at -20°C for 2 h after neutralisation with 0.855M Tris-base.

Reverse phase chromatography (Sep-Pak C18) extraction was performed as described by Goddard *et al.* (1988). Briefly, plasma (200 μ l) and culture media samples (5ml) were acidified with 800 μ l or 100 μ l of 2M HCl respectively and incubated for 30 min. Sep-Pak C18 cartridges were prepared by sequential washes with 5 ml isopropanol, 5ml methanol and 10 ml acetic acid (4%). Samples were loaded into cartridges and IGFBPs eluted with 10 ml of 4% acetic acid. IGF-I was eluted with 5 ml methanol and following evaporation at 37 °C was dissolved in 1 ml assay buffer.

Acidification of plasma or serum samples was achieved by adding 10 μ l of 0.5M phosphoric acid to 100 μ l of sample. After adjusting the volume to 500 μ l with IGF-RIA acid buffer (0.02M sodium phosphate, 0.1M NaCl, 0.1% BSA, 0.02% NaN₃, 0.1% Triton X-100; pH 2.8) the samples were incubated overnight at 4°C before assay. Culture samples (5 ml) were acidified by adding 100 μ l of 0.5M phosphoric acid and incubating overnight at 4°C.

Size exclusion column chromatography

Low pressure acid gel chromatography was performed according to the method of Hintz and Liu (1977), with slight modifications. Samples were chromatographed using Sephadex G75 (Pharmacia, Uppsala, Sweden) in a 1.6cm x 75cm glass column equilibrated with IGF-RIA acid buffer (pH 2.8), pumped up the column at 0.3 ml min⁻¹. The column was calibrated with blue dextran, cytochrome C and ¹²⁵I-IGF-I

(10^6 cpm). One millilitre of either acidified serum or conditioned culture-medium (10 times concentrated by ultrafiltration; Amicon Ltd., Upper Mill, Stonehouse, Gloucestershire, UK) was loaded on to the column and 750 μ l fractions collected. Fractions containing IGF and IGFBP were identified by RIA.

Acid gel high performance liquid chromatography (HPLC) was performed according to the protocol of Owens *et al.* (1990) with slight modifications. An HPLC Protein-Pak 125 (7.8 x 300 mm) column (Waters, Division of Millipore, Milford, MA 01757, USA) was used in a Waters 600E HPLC system, fitted with a pre-column filter packed with the same material as the column. Non-specific binding sites in the column were blocked by flushing the column with the mobile phase (0.2 mol/l acetic acid containing 0.05 mol/l trimethylamine and 0.05% Tween-20, pH 2.5) containing 0.1% BSA overnight. Prior to sample application, the column was washed with the mobile phase without BSA for a further 4 h to ensure the complete removal of free BSA. Plasma samples were prepared by diluting 100 μ l of plasma in 400 μ l water, the mixture was acidified with 150 μ l of sample treatment buffer (0.8 mol/l acetic acid containing 0.20 mol/l trimethylamine and 0.05% Tween-20; pH 2.5). After a 30 min incubation, 600 μ l of chloroform was added and the samples centrifuged at 10,000 X g for 10 min at 4 °C. The upper fat-extracted layer was recovered and filtered through a 0.45 μ m filter (Whatman Inc. Clifton, NJ, USA). Conditioned culture media (2.5 ml) was concentrated by lyophilisation (Edwards Freeze dryer, Modulyo, Edwards High Vacuum, Crawley, UK) and subsequently resuspended in 250 μ l of water. This sample was acidified with 100 μ l of sample treatment buffer and filtered before loading on to the column. Acidified samples were applied with an automated injector (Waters 712 WISP), and the mobile phase pumped at 0.5 ml/min for 30 min. Fractions (250 μ l) were neutralised by the addition of 100 μ l of 0.4M Tris-base. Fractions containing either IGFBP or IGF were determined by IGF-I radioimmunoassay and compared with the elution profile of 125 I-IGF-I.



2.10 WESTERN LIGAND BLOT

Residual IGFBPs from extracted and non-extracted plasma and culture media were analysed by SDS:PAGE on a 12% homogeneous gel, under non-reducing conditions in a discontinuous buffer system (Hossenlopp *et al.*, 1986). EAA and EAA-C extracted samples were dialysed against 0.1M Tris buffer and concentrated by ultrafiltration before loading (100 µl) on to the stacking gel. Gels were electroblotted on to 0.2 µm nitro-cellulose membranes (Anderman & Co. Ltd. Kingston-upon-Thames, Surrey, UK), with a semi-dry blotter (Semiphor TE77, Hoeffer Scientific Instruments. San Francisco, Ca. USA) according to the manufacturer's instructions. After protein transfer, the membrane was blocked (0.1% BSA), dried at room temperature, probed with ¹²⁵I-IGF-I and washed according to the method described by Hossenlopp *et al.* (1986). After allowing the blot to dry IGFBPs were detected by exposure to Kodak X-omatic film at -70°C with intensifying screens for 10 days.

2.11 RT-PCR

2.11.1 RNA EXTRACTION.

Total RNA was extracted from cultured cells using RNeasy kits (Qiagen Ltd. Dorking, Surrey). Briefly, after removal of the culture medium, cells were scraped out of the culture flask (Nunc) and harvested into a 1 ml sterile Eppendorf tube. Cells were centrifuged (5 seconds) at 10,000 X g and the remaining culture medium removed followed by the addition of 600 µl of lysis buffer. Cell lysates were stored at -80°C until samples from three cultures were collected when RNA was extracted simultaneously from all samples according to manufacturer's instructions (Qiagen Ltd.). The quantity and purity of total RNA was estimated by absorbance at 260 nm (A_{260} of 1 was equivalent to 40 µg/ml) and 260/280nm ratio (>1.7) and stored for analysis at -80°C.

2.11.2 REVERSE TRANSCRIPTION

Total RNA (0.1 mg in 10 μ l of DEPC-treated water) was mixed with 0.5 μ l dNTP (125 pmol; Promega Ltd. Southampton, UK) and 0.5 μ l RNasin (Promega), denatured at 70°C for 10 min and cooled immediately to 4°C. The denatured RNA was reverse transcribed by adding 9 μ l of RTase mix (see Appendix) and incubated at 20°C for 10 min followed by 42°C for 60 min. Following the reaction, transcriptase activity was terminated by heating at 95°C for 5 min, and 20 μ l of dilution buffer (see Appendix) was added and stored at -80°C until further use. Control samples were made as described above, but omitting the RTase (Superscript II, RNase H⁻ reverse transcriptase; Life Technologies. Gibco) from the reaction mix.

2.11.3 POLYMERASE CHAIN REACTION.

A 2.5 μ l aliquot from the RTase reactions (samples containing the cDNA and controls) were mixed with 1.5 μ l 10X PCR buffer (500 mM KCl and 200 mM Tris-HCl pH 8.4; Gibco), 14 μ l dH₂O, 1 μ l primer mix (approximately 10 pmol of upstream and downstream primers) and 1 μ l Taq polymerase (1:5 dilution; Gibco). The cDNA in the mixture was amplified for 30 cycles (93°C, 65°C and 72°C for 30 seconds each) after which the reaction was continued at 72°C for 4.5 min. The products were visualised by electrophoresis of 5 μ l of the reaction sample on a 4% Nusieve GTG agarose gel (Flowgen) stained with ethidium bromide and bands visualised under ultraviolet light.

2.12 ELECTRON MICROSCOPY

Bovine granulosa cells from medium-sized follicles were cultured as described above. After 144 h of culture, medium was removed and the cells were fixed *in situ* with 2.5% glutaraldehyde in 0.075 M sodium cacodylate (pH 7.4; Agar Scientific, Stansted, Essex, UK) at room temperature for 1 h. Following fixation, samples were washed twice with 0.075 M sodium cacodylate containing 0.2 M sucrose (pH 7.4) and postfixed in 1% osmium tetroxide in 0.175 M sodium cacodylate (1:1 dilution of

2% osmium tetroxide in distilled water with 0.35 M sodium cacodylate; pH 7.4) for a further 45 min and dehydrated in increasing concentrations of ethanol (50, 60, 80, 90 100 % ethanol for 15 min each). After dehydration, samples were pre-embedded in 50:50 v/v LR-White (London Resin Co. Basingstoke, Hampshire, UK): ethanol mixture for one hour, followed by 100% LR-White overnight. LR-White was changed again and polymerised overnight at 60 °C. The resulting acrylic disks containing the cultured cells were cut into 2 mm wide strips and selected strips were re-embedded in beam capsules (Agar Scientific).

Samples were trimmed with glass knives and orientated for ultramicrotomy. Ultrathin sections (50-60 nm; silver-gold interface) of granulosa cell cultures were cut perpendicular to the plating surface. Sections were floated in water, mounted on 200 mesh copper grids and counter-stained for 5 min with uranyl acetate (2% uranyl acetate in 50% ethanol; Watson, 1958) and 7 min with lead citrate (Reynolds, 1963). Ultra-structural observations were made using an Phillips EM300 electron microscope (Eindhoven, Netherlands).

2.13 STATISTICAL ANALYSES

Details of the specific statistical models applied for analyses of the data are described in the appropriate chapters. Statistical analyses were performed using either Genstat 5 (Rothamstead Experimental Station) or SAS (SAS Institute Inc., Cary, NC, USA. Release 6.10) statistical packages.

3. INSULIN-LIKE GROWTH FACTOR-I (IGF-I)-EXTRACTION AND MEASUREMENT.

3.1 ABSTRACT

IGFBP extraction protocols were tested for their efficacy in removing IGFBPs from bovine plasma and bovine granulosa cell culture medium compared to standard acid exclusion chromatography. Traditional extraction methods (acidification, Sep-Pak, ethanol:acetone:acetic acid (EAA) and EAA-cryoprecipitation (EAA-C)), failed to remove all the IGFBPs from both granulosa cell culture medium and plasma. However, EAA and EAA-C treatment of plasma samples gave values similar to those obtained by acid high performance liquid exclusion chromatography (HPLC) after correction for extraction efficiency. There was an inverse relationship between IGF-I concentration in plasma samples, as measured using HPLC, and IGF-I concentration after EAA extraction. Furthermore, the interference caused by residual IGFBPs differed between samples taken from animals given various treatments that altered peripheral IGF-I concentrations.

EAA was the most effective extraction method for culture media and plasma samples, but residual IGFBPs caused an overestimation of IGF-I concentrations by RIA. In culture media (but not plasma) it was possible to block the interference of IGFBPs in the IGF-I assay in both extracted and non-extracted culture samples by the addition of excess IGF-II. Using this assay procedure, IGF-I production by bovine granulosa cells was undetectable. This was confirmed by HPLC acid chromatography.

It is concluded that HPLC extraction is essential for the accurate measurement of peripheral IGF-I concentrations. For granulosa cell culture media, it is possible to measure IGF-I concentrations in non-extracted samples if the IGFBPs are blocked by adding IGF-II. Using either this assay, or HPLC acid chromatography, IGF-I was undetectable in culture media, suggesting that IGF-I is not produced by non-luteinised bovine granulosa cells.

3.2 INTRODUCTION

Insulin-like growth factor (IGF) -I and -II are potent stimulators of cell proliferation and differentiation. The liver is the major source of IGF-I. However, it is also produced in many other tissues (Lund, 1994). IGF-II is expressed by a range of tissues, and is thought to play a major role in foetal growth and development (Giudice, 1992).

In biological fluids and cell culture medium, IGFs are bound to specific binding proteins (Hossenlopp *et al.*, 1986; Jones and Clemmons, 1995). IGF-binding proteins (IGFBPs) bind both IGF-I and -II competitively (Hossenlopp *et al.*, 1986; Blum *et al.*, 1988), and must be removed before their measurement. The problems encountered when measuring IGF concentrations have been highlighted recently (Lee and Henricks, 1990; Breier *et al.*, 1991; Bang, 1994; Frey *et al.*, 1994; Holly and Hughes, 1994; Rivero *et al.*, 1994). However, there is still a requirement for further improvement of IGF assays, and for the development of reliable procedures for the rapid removal of IGFBPs from biological samples.

It is important that extraction protocols are validated before use. Such validation should include: i) parallelism between the standard and the extracted sample, ii) specificity of the antibody and iii) demonstration of the absence of IGFBPs in the assay sample (Bang, 1994).

Although size exclusion chromatography under acid conditions is acknowledged to be the best method for IGFBP extraction (Blum and Breier, 1994), this procedure is time consuming and other more practical methods have been developed which allow a larger sample throughput. These approaches include ethanol:acetone:acetic acid (EAA) extraction (Enright *et al.*, 1989), a modification of the acid:ethanol extraction procedure (Daughaday *et al.*, 1980) and reverse phase column chromatography (Sep-Pak C18) (Goddard *et al.*, 1988, Frey *et al.*, 1994). Cryoprecipitation (-20 °C for 1h) also improved the efficiency of extraction after acid:ethanol sample treatment (Breier *et al.*, 1991), but has not been fully evaluated after EAA extraction.

The ovarian follicle is a site of action of IGF-I and -II and specific receptors for both ligands have been found in follicles (Adashi, 1992a; Hammond *et al.*, 1991). Chapter

6 describes the development of a serum-free culture system for bovine granulosa cells in which the follicular phenotype of the cells is maintained without significant luteinisation. In this culture system, bovine granulosa cells respond to IGF-I stimulation with increased steroidogenesis and proliferation (Chapter 6). In addition, IGF-BPs are also produced by bovine granulosa cells and may regulate IGF-I action locally (Armstrong *et al.*, 1996c).

Unlike the rat, it is unclear whether ruminant granulosa cells produce IGF-I. Evidence for IGF-I production by ovine granulosa cells has been contradictory. A recent report using *in situ* hybridisation suggested that ovine granulosa cells express IGF-I mRNA (Leeuwenberg *et al.*, 1995). However, using similar techniques other researchers have been unable to detect IGF-I mRNA (Perks *et al.*, 1995).

The objectives of the work described in this chapter were i) to evaluate the use of a competitive IGF assay for measuring IGF-I concentrations in bovine plasma and serum and granulosa cell culture media samples, ii) to assess the effects of residual IGF-BPs and compare this method with the HPLC extraction method, and iii) to determine whether IGF-I is produced by non-luteinised bovine granulosa cells *in vitro*.

3.3 MATERIALS AND METHODS

3.3.1 SAMPLES

Plasma samples from mature Hereford-Friesian heifers were collected as described in Chapter 2. Serum samples from animals treated with bovine somatotrophin (BST, 25 mg daily; Somidobove, Eli Lilly & Co. Indianapolis, IN) were kindly provided by Dr. Jin Gong (Gong *et al.*, 1993a). Plasma and serum samples were stored at -20°C until assayed.

Culture medium was obtained from serum-free cultures of bovine granulosa cells as described in Chapter 2. For assessment of extraction protocols, $3-5 \times 10^6$ cells were cultured in 10 ml of McCoy's 5a medium supplemented with 10 ng/ml insulin, and 10 ng/ml Long-R3 IGF-1 and 1 ng/ml bFSH in a 50 ml tissue culture flask. Additionally

75×10^3 viable granulosa cells/well were seeded into 96 well plates containing 250 μ l of medium, supplemented with 10 ng/ml insulin and 1 ng/ml bFSH. The cells were cultured as described previously for up to 6 days. Cultured media were stored at -20°C until assayed.

3.3.2 IGF EXTRACTION PROTOCOLS AND ANALYSIS

IGF from both plasma and culture samples was extracted by either EAA, EAA plus cryoprecipitation (EAA-C), reverse phase chromatography (Sep-Pak C18) or by acidification methods (see Chapter 2) and fractions were analysed by IGF-I RIA. In addition, samples were processed by low- or high-pressure size exclusion column chromatography and fractions containing either IGFBP or IGF were determined by IGF-I RIA and compared to the elution profile of 125 I-IGF-I. A competitive IGF RIA in which interference by IGFBPs was blocked with excess IGF-II, was used to evaluate the efficiency of EAA and measure IGF-I in non-extracted culture samples. The intra- and inter-assay coefficients of variation were 8% and 13% respectively.

Residual IGFBPs from extracted and non-extracted plasma and culture media were analysed by western ligand blot.

3.3.3 STATISTICAL ANALYSES

Differences in the recovery efficiency of the extraction protocols were evaluated by ANOVA, with Bonferroni's modified *t*-test to evaluate differences between extraction methods. Student's *t*-test was used to evaluate differences in recovery efficiency. The parallelism between displacement curves of extracted samples and standards was tested by analysis of homogeneity of regression, after logarithmic transformation of the volume (μ l) or concentration of standard (IGF-I; pg/tube) added to the assay tubes. The effect of extraction procedure on the amount of IGF-I detected by RIA relative to that detected after HPLC (total IGF-I) was analysed by ANCOVA using total IGF-I as a covariate. Regression analysis was used to evaluate the effect of the addition of IGF-II into the RIA of EAA extracted plasma samples.

3.4 RESULTS

3.4.1 SIZE EXCLUSION COLUMN CHROMATOGRAPHY

Using low pressure acid gel chromatography (Figure 3.1a), the elution volume for native IGF-I (87ml), coincided with that for ^{125}I -IGF-I. For plasma samples (Figure 3.1b), several peaks of immunoreactivity were observed after acid gel chromatography, eluting between 48 and 63 ml which was detected as apparent IGF-I immunoreactivity in the IGF-I RIA. In contrast, only a single peak of IGFBPS was detected in bovine granulosa cell culture medium (Figure 3.1c).

Acid exclusion HPLC also separated IGFBPs from IGF-I (Figure 3.2). IGFBPs and IGF-I eluted between 7 to 8.5 ml and 9 to 11 ml respectively. As observed with low pressure acid gel chromatography, the elution of the second peak of IGF-I immunoreactivity coincided with the elution volume of ^{125}I -IGF-I. Recovery of ^{125}I -IGF-I was 99%. Thus, the HPLC procedure was the method of choice, since it was less time consuming than low pressure chromatography (0.5 vs 6h per sample). Moreover, using an automated sample injector, up to 40 samples per day could be processed. Hence, HPLC extraction was used as the standard against which other extraction protocols were compared.

3.4.2 DISPLACEMENT CURVES FOR EXTRACTED SAMPLES AND IGF-I STANDARD

Regression analysis of IGF-I standard and plasma IGF-I after HPLC extraction (Figure 3.3a), EAA extraction, EAA-C extraction (Figure 3.3c), or Sep-Pak extraction (Figure 3e) showed no differences in slopes (regression slope = -42.1 ± 1.5 ; $p > 0.05$) between the displacement curves. The slopes of the inhibition curves of plasma following acidification (slope = -16.9 ± 2.1 ; Figure 3.3b), and granulosa cell culture medium after acidification (slope = -71.9 ± 5.2 ; Figure 3.3b), EAA extraction (slope = -83.8 ± 6.2 ; Figure 3.3d), EAA-C extraction (slope = -65.7 ± 6 ; Figure 3.3d) or Sep-Pak extraction (slope = -55.4 ± 2.5 ; Figure 3.3e) differed significantly ($p < 0.05$) from the slope of the IGF-I standard. However, HPLC-extracted IGFBPs from both plasma and granulosa cell culture medium (Figure 3.3f), produced similar displacement curves to that produced by the IGF-I standard curve ($p > 0.05$).

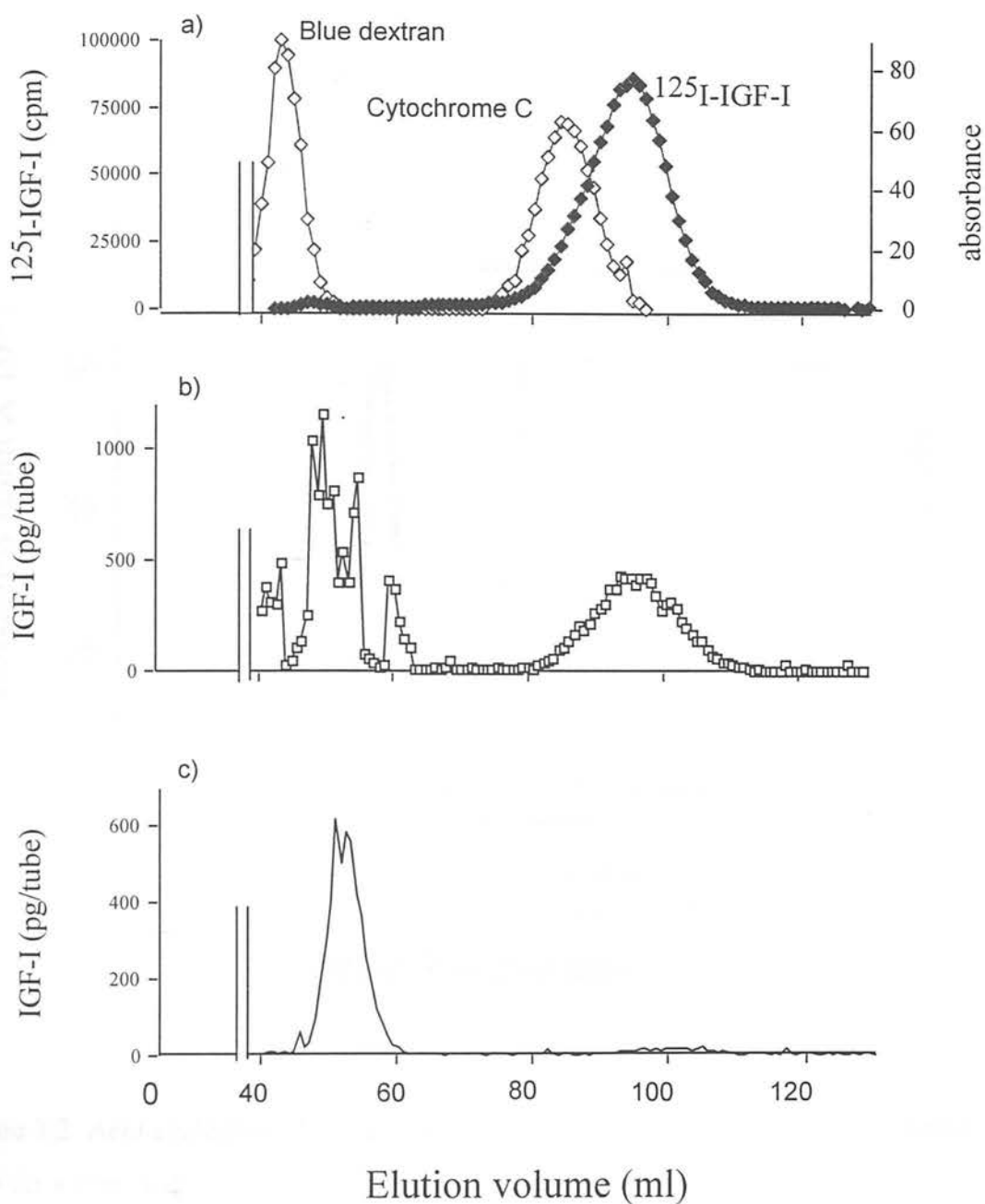


Figure 3.1. Low pressure acid gel column chromatography using Sephadex G75. a) Molecular weight standards, b) bovine plasma and c) granulosa cell culture medium. Chromatography was performed using a column (1.6cm x 75cm) equilibrated with IGF acid buffer (0.02M sodium phosphate, 0.1M NaCl, 0.1% BSA, 0.02% NaN_3 , 0.1% Triton X-100; pH 2.8) and pumped at 0.3 ml/min.

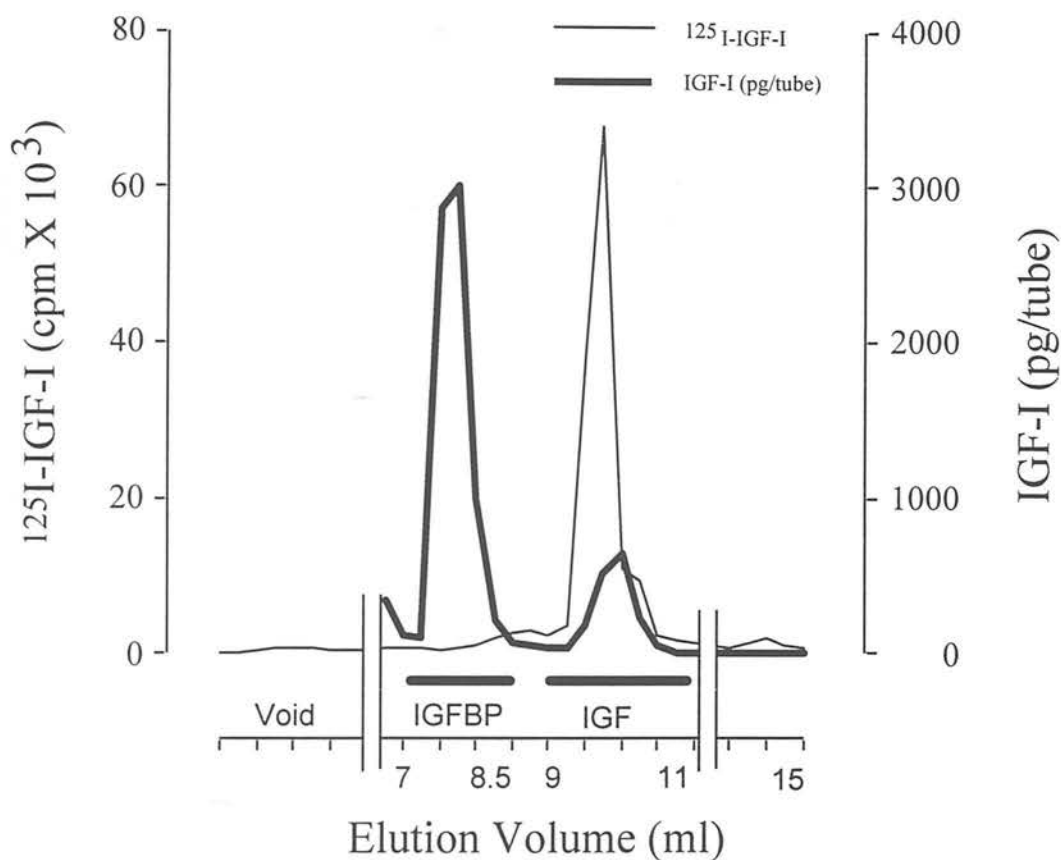
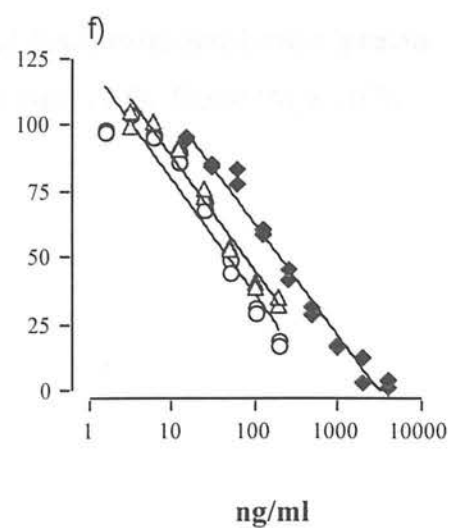
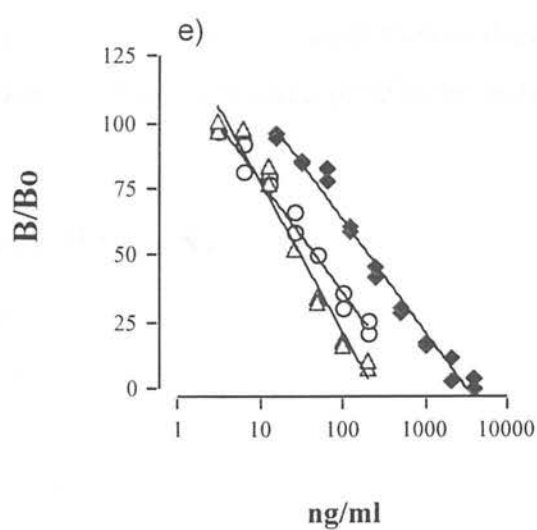
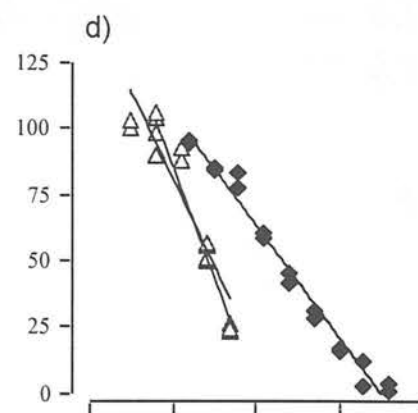
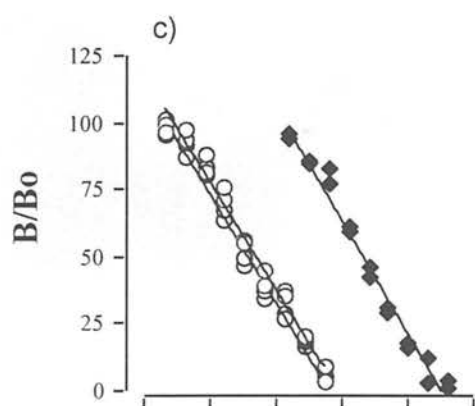
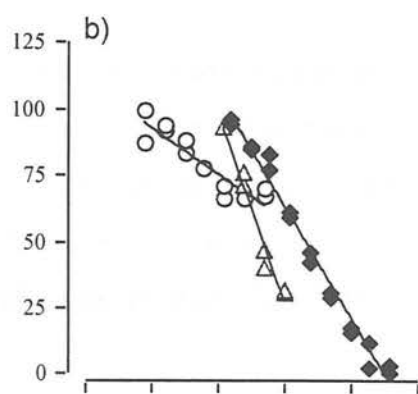
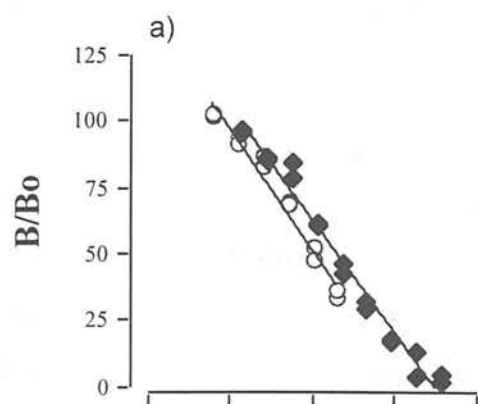


Figure 3.2. Acid exclusion HPLC of bovine plasma (solid line) and ^{125}I -IGF-I (dotted line) on a Protein-Pak 125 (7.8 x 300 mm) column eluted with 0.2 mol/l acetic acid containing 0.05 mol/l trimethylamine and 0.05% tween-20 (pH 2.5) at 0.5 ml/min for 30 min.

Figure 3.3. Comparison of displacement curves for IGF-I standard (◆) plasma IGF-I samples (○) or culture medium extracts (▲): isolated by HPLC, (a); acidified culture and plasma samples, (b); ethanol:acetone:acetic acid, (EAA) and EAA-plus cryoprecipitation extracted plasma samples, (c); EAA and EAA-C extracted culture media, (d); plasma and culture media extracted by Sep-Pak, (e) and HPLC-isolated IGFBP from plasma and granulosa cell culture media samples, (f).



3.4.3 ¹²⁵I-IGF-I RECOVERY EFFICIENCY

For both plasma and culture medium, EAA extraction (77.4%) had a higher ($p<0.05$) recovery efficiency compared to Sep-Pak extraction (39.4%). Cryoprecipitation did not improve EAA extraction efficiency ($p>0.05$). Recovery of ¹²⁵I-IGF-I did not differ ($p>0.05$) between plasma and culture medium in any of the protocols tested. However, the recovery of ¹²⁵I-IGF-I differed significantly ($p<0.05$) from 100% for EAA, EAA-C and Sep-Pak extractions.

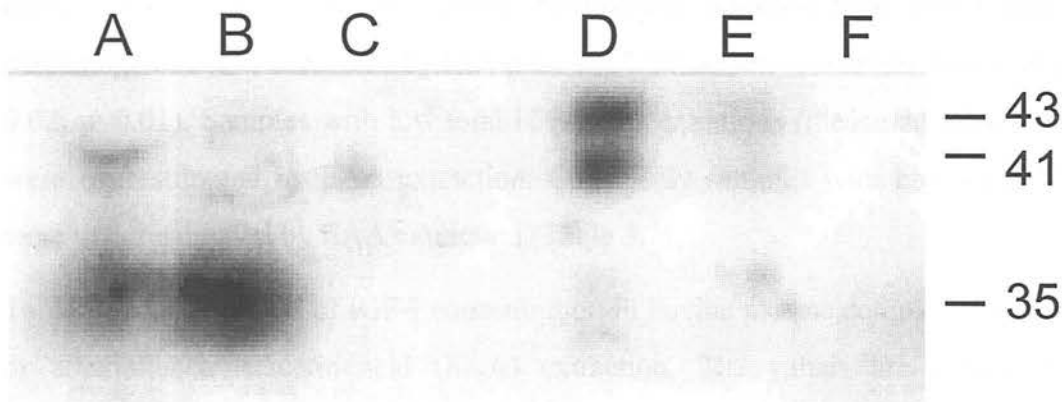


Figure 3.4. Autoradiography of a ¹²⁵I-IGF-I ligand blot of unconcentrated bovine granulosa cell culture medium (lane A), Sep-Pak extracted culture medium (lane B), ethanol:acetone:acetic acid-extracted culture medium (lane C), bovine plasma (lane D), Sep-Pak extracted plasma (lane E) and EAA extracted bovine plasma (Lane F). Size of binding proteins are indicated on right of the figure ($M_r \times 10^{-3}$).

3.4.4 WESTERN LIGAND BLOT

Samples of plasma and culture media differed markedly in IGFBP content (Figure 4). Whilst plasma contained mainly high molecular mass binding proteins (lane D; 43, 41 kDa doublet), granulosa cell culture media contained lower molecular weight IGFBPs (lane A; 35 kDa). EAA extraction appeared to eliminate all IGFBPs from both culture medium (lane C) and plasma (lane F). In contrast, discrete amounts of IGFBP were detected in Sep-Pak extracted samples (lane B and E).

3.4.5 MEASUREMENT OF IGF-I AFTER EXTRACTION

IGF-I concentrations in bovine plasma samples after HPLC extraction were significantly higher ($p<0.05$) than IGF-I concentrations in samples extracted by other procedures (Table 3.1). However, after EAA extraction and correction for recovery efficiency, the mean IGF-I concentration did not differ from the mean IGF-I concentration after HPLC ($p>0.05$). Despite the lack of difference in mean IGF-I concentrations, there was an inverse relationship between total IGF-I and the percentage of IGF-I-measured by RIA after EAA extraction (covariate slope= -0.08 ± 0.02 ; $p<0.01$). Samples with low total IGF-I concentrations (measured after HPLC), were overestimated by EAA extraction. Conversely samples with high total IGF-I were underestimated by EAA extraction (Table 3.2).

Table 3.2. Comparison of IGF-I concentration in bovine plasma samples after HPLC or ethanol:acetone:acetic acid (EAA) extraction. The values are corrected for recovery efficiency. The percentage of IGF-I detected after EAA extraction relative to concentrations after HPLC are also given.

Sample	HPLC (ng/ml)	EAA (ng/ml)	EAA (% of HPLC)
1	197	242	123
2	248	251	101
3	297	263	88
4	320	308	96
5	502	387	77

Table 3.1. Comparison of IGF-I concentrations in bovine plasma (n=5) and granulosa cell conditioned media (n=5) following HPLC, acidification, Sep-Pak, ethanol:acetone:acetic acid (EAA) extraction or EAA-cryoprecipitation. Total IGF-I measured after HPLC was used as the standard to which other extraction protocols were compared. The results are expressed as ng/ml after correction for recovery efficiency, and as a percentage of the IGF-I detected relative to HPLC. ** p<0.01, n.d.= non-detectable.

	HPLC	Acidification	Sep-Pak	EAA	EAA-C
Plasma (ng/ml)	312.9± 51.6	30 ± 6.2**	46.4 ± 16.4**	291 ± 26.7	280.7±24.2
% of HPLC	100	9.4 ± 5.9 **	14.7 ± 4 **	97.3 ± 7.5	94 ± 6.8
Range (%)		(8-10)	(0-23)	(77-123)	(73-115)
Culture media					
(ng/ml)	n.d.	8.7 ± 1.2**	2.1 ± 0.9**	14.4± 0.6**	7.2 ± 2.9**

3.4.6 MEASUREMENT OF IGF-I IN GRANULOSA CELL CULTURE MEDIA AND PLASMA USING A COMPETITIVE IGF-I RIA

The effects of residual IGFBPs and the addition of increasing amounts of IGF-II (0-4 ng/ml) were evaluated in an IGF-I competitive RIA. Figure 3.5 shows the effect of the addition of IGF-II on apparent IGF-I concentrations in granulosa cell culture conditioned media. EAA-extraction reduced the interference of the IGFBPs by at least 90%. The addition of IGF-II to either extracted or non-extracted culture medium (Figure 3.5) caused an apparent decline in the amount of IGF-I present in the sample. This decline reached a nadir at around 0.5 ng of added IGF-II per tube for extracted samples and at 2 ng/tube of added IGF-II in non-extracted culture samples (Figure 3.5). Furthermore, at the lowest point of the curve, the concentration of added LR3-IGF-I measured in non-extracted (19.32 ± 4.6 pg/tube) and extracted samples (19.5 ± 0.3) did not differ ($p > 0.05$).

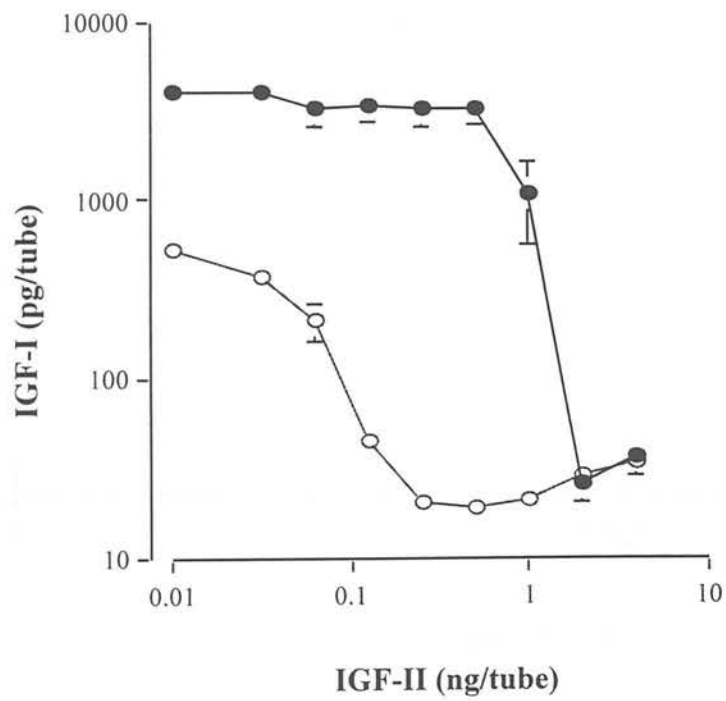


Figure 3.5. Effect of the addition of IGF-II (0-4 ng/tube) on apparent concentration of IGF-I in ethanol:acetone:acetic acid extracted (○) and non-extracted (●) granulosa cell culture media. Mean \pm SEM.

Plasma samples from BST-treated animals had significantly higher ($p<0.01$) IGF-I concentrations than plasma from untreated animals. Increasing amounts of IGF-II in the assay tubes increased the apparent IGF-I concentration. However, the addition of

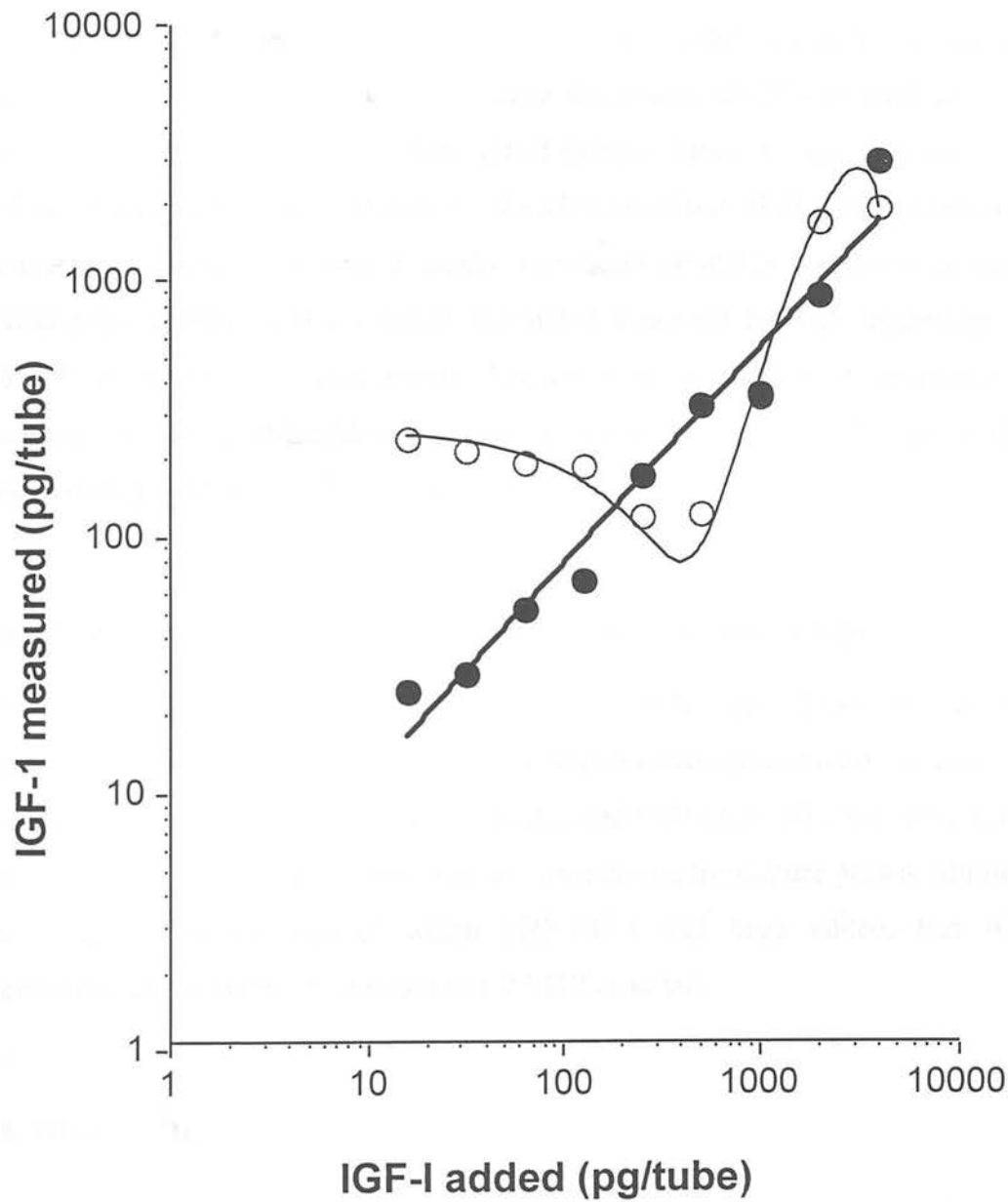


Figure 3.6. The effects of the presence (●) or absence (○) of IGF-II in blocking the interference of IGFBPs produced by granulosa cells in an IGF-I radioimmunoassay.

IGF-II into the BST-treated sample tubes resulted in a significantly greater ($p<0.01$) increase in apparent IGF-I concentration (regression slope= 36.9) compared to the apparent IGF-I concentration in samples from untreated animals (slope=13).

Based on the displacement of IGF-I by IGF-II in extracted and non-extracted culture media, IGF-I could be measured in non-extracted culture medium by blocking IGFBP interference with IGF-II. To validate this assay, IGFBPs isolated after acid gel chromatography (Figure 3.6) were added to assay tubes in a quantity equivalent to 50 μ l of culture medium, followed by the addition of IGF-II (2 ng/tube) and IGF-I measured by RIA. There was a highly significant ($P<0.01$) positive relationship between the amount of IGF-I added and IGF-I measured by RIA (regression: $y=1.65x^{0.86}$; $r^2=0.96$). In contrast, when IGFBPs were not blocked, high apparent IGF-I concentrations were obtained, even when no IGF-I was added to the assay tubes (regression: $y=265.6 - 0.965x + 10^{-3}x^2 - 10^{-7}x^3$; $r^2=0.99$).

3.4.7 IGF-I PRODUCTION BY BOVINE GRANULOSA CELLS IN CULTURE

Using the competitive RIA described above, non-extracted culture samples were analysed for their IGF-I content. Bovine granulosa cells from small, medium and large follicles cultured with insulin (10 ng/ml) and FSH (1ng/ml) for 6 days did not produce detectable amounts of IGF-I at any time during the culture period. Similarly, cells cultured in medium to which LR3-IGF-I had been added, had IGF-I concentrations similar to the amount of LR3-IGF-I added.

3.5 DISCUSSION

We have highlighted problems in some of the current methods used to measure IGF-I concentrations in bovine plasma and granulosa cell culture medium which were the result of insufficient extraction of IGFBPs from the samples before RIA. To circumvent these problems, two procedures were developed which were effective in reducing IGFBP interference in the IGF-I RIA. It was shown that, 1) HPLC extraction was the only extraction procedure capable of eliminating the IGFBP

interference in both peripheral blood samples and culture medium samples, 2) IGF-I could be measured in non-extracted bovine granulosa cell culture medium using an IGF competitive assay and 3) non-luteinised bovine granulosa cells did not produce measurable levels of IGF-I.

The competitive IGF-I RIA allowed measurement of IGF-I from non-extracted bovine granulosa cell culture medium. When this assay was used to measure IGF-I in granulosa cell culture samples from small, medium and large follicles, IGF-I concentrations were below the detection limit of the assay. These results do not support the hypothesis proposed for the mouse that IGF-I is an autocrine factor involved in the control of granulosa cell function (Adashi and Rohan, 1992). Two possible explanations exist for this lack of IGF-I production. Firstly, the expression of IGF-I declines once the cells are in culture. Botero *et al.* (1993) demonstrated that rat granulosa cell IGF-I mRNA expression declined after only 3 hours in culture. Secondly, unlike the rat, bovine granulosa cells do not produce IGF-I. Although IGF-I production by bovine granulosa cells *in vitro* has been reported (Spicer *et al.*, 1993b), IGF-I measurements were made on samples after acid ethanol extraction, and as we have shown in this study, residual IGFBPs not removed by extraction (Figure 5) gave false positive results. In addition, Wathes *et al.* (1995) did not detect IGF-I in ovine granulosa cell culture conditioned-medium until the cells luteinised. IGF-I mRNA expression in bovine granulosa cells *in vivo* has also been reported (Spicer *et al.*, 1993b). However, the animals used in this latter study had been treated with a superovulatory hormonal regime. Therefore, a certain degree of luteinisation of the follicles after this treatment would be expected, and both IGF-I peptide (Amselgruber *et al.*, 1994) and mRNA expression (Einspanier *et al.*, 1990) have been detected in bovine luteal cells. More recently, Perks *et al.* (1995) were unable to find IGF-I mRNA expression in granulosa cells of ovine follicles using *in situ* hybridisation, but IGF-I mRNA was found in granulosa-derived cells in the corpus luteum. Our results provide evidence for the lack of IGF-I production by non-luteinised bovine granulosa cells *in vitro*.

For plasma samples, all the extraction methods evaluated (except acid gel chromatography) were unsuitable for the complete removal of IGFBPs. To monitor the effects of the residual amounts of IGFBPs remaining after EAA extraction, a competitive IGF-I RIA was used together with the addition of excess IGF-II to displace IGF-I from IGFBPs. The addition of IGF-II into the assay tubes resulted in an increase in the apparent IGF-I concentration (Figure 3.5). This observation was interpreted as due to the underestimation of the total IGF-I present in the sample, because of IGFBP interference in the assay. The addition of IGF-II blocked binding protein interference, leaving IGF-I free to react with the antibody. This result was comparable to that found for IGF-II by Blum *et al.* (1988), though in this study, the addition of IGF-II caused an increase in IGF-I. The IGF-I values obtained in the presence of IGF-II, used as an IGFBP-blocker, depended on the molar ratio of IGF-I : IGFBP present in the sample after extraction. If this ratio was < 1 the addition of IGF-II produced a curve with a negative slope. If the ratio was > 1 , IGF-II addition produced a curve with a positive slope (Figure 3.5). We therefore conclude that in EAA-extracted bovine plasma, IGF-I was present in a molar excess to IGFBP, resulting in an underestimation of IGF-I concentrations.

There was also evidence for variation in EAA extraction efficiency between samples from animals under different treatments or physiological conditions. When comparing the effects of IGF-II on EAA-extracted samples from animals treated with BST (which increases IGF-I concentrations: Gong *et al.*, 1993a), with extracted samples from untreated animals, the regression curve for IGF-I levels in the presence of increasing amounts of IGF-II differed ($p < 0.01$) between treated and untreated animals. Not only did treated animals have higher apparent IGF-I concentrations ($p < 0.01$), but addition of IGF-II produced a greater displacement of IGF-I from the IGFBPs ($p < 0.01$) than with untreated animals. This suggests increased amounts of IGFBPs in extracted samples from BST-treated animals compared with untreated animals. This is probably due to BST stimulation of hepatic IGFBP production (Lund 1994; Jones and Clemmons, 1995). Indeed, it is known that as well as increasing circulating IGF-I concentrations, BST increases IGFBP-3, and decreases IGFBP-2

concentrations (McGuire *et al.*, 1992). These same authors reported that metabolic status affected the ratio between IGFBP-1, IGFBP-3 and IGF-I.

Furthermore, total IGF-I concentration after HPLC extraction was inversely related to the percentage of IGF-I detected after EAA extraction. Whether this result is due to qualitative or quantitative changes in residual IGFBPs, or to differences in the recovery of IGF-I by the extraction protocol, could not be determined in this study. We conclude that for plasma samples, acid gel chromatography extraction must be used for the reliable measurement of total IGF-I.

In the case of granulosa cell cultures, the interference of non-extracted samples by IGFBPs were blocked by the addition of IGF-II (Figure 3.5).

Recovery efficiencies of ^{125}I -IGF-I from plasma samples after EAA and EAA-C extraction (71.4 and 75.6%) did not differ ($p>0.05$), and corresponded to those found by EAA-extraction of bovine (Lee and Henricks, 1990) and ovine plasma samples (Breier *et al.*, 1991), but were significantly lower than the 100% recovery efficiency reported by Enright *et al.* (1989). In contrast, recoveries by Sep-Pak extraction were highly variable and low (Table 3.1). Comparison of total IGF-I levels measured after HPLC with measurements after extraction by other methods yielded varying results. In plasma, all extraction procedures produced lower apparent IGF-I concentrations than HPLC extraction. After correcting for recovery efficiency EAA and EAA-C extracted plasma samples did not differ in mean IGF-I concentration with levels measured after HPLC extraction. However, how IGF-I recovery is affected by the ratio of IGF:IGFBP needs further investigation.

Parallelism between curves of extracted samples to the standard is interpreted as an absence of interference from binding proteins. For plasma samples, all extraction procedures produced parallel displacement curves with the IGF-I standard. Parallelism of extracts containing residual amounts of IGFBPs has been reported (Blum *et al.* 1988; Lee and Henricks, 1990), and isolated IGFBPs gave parallel displacement curves to IGF-I (Lee and Henricks, 1990). Displacement curves of extracted culture media samples were not parallel to IGF-I standard, however. These results were not due to lack of interference of IGFBPs in the assay, since IGFBPs

isolated by HPLC also produced curves parallel to IGF-I standard (Figure 3.3). We hypothesise, therefore, that following neutralisation, IGF binding to IGFBP reaches equilibrium and the free IGF-I is detected in the assay. However, excess IGFBPs interfere in the IGF RIA giving parallel displacement curves.

Ligand blot analysis is the most commonly used method to determine directly the presence or absence of IGFBPs after extraction. As expected, the major binding protein detected in plasma (41-43 kDa) was identified as IGFBP-3 (Jones and Clemmons, 1995), but other binding proteins of lower molecular mass (35 kDa) were also seen. In contrast, culture medium contained only low molecular mass IGFBPs. This pattern of IGFBP production is similar to that in conditioned culture medium from ovine granulosa cells (Armstrong *et al.*, 1996b) and with the pattern of IGFBP mRNA expression in bovine granulosa cells (Armstrong *et al.*, 1996c). Sep-Pak extraction was ineffective in removing all IGFBPs from either plasma or cell culture samples, whereas EAA and EAA-C extraction appeared to remove IGFBPs completely. However, ligand blot analysis may not be sensitive enough to detect the small amounts of IGFBPs remaining after these extraction procedures.

In conclusion, the reliable measurement of total plasma IGF-I requires IGFBP extraction by size exclusion acid chromatography. For bovine granulosa cell cultures, it was possible to measure IGF-I in non-extracted samples using a competitive RIA, with the addition of IGF-II (2ng/tube). No IGF-I production by bovine granulosa cells in culture could be detected, questioning previous results that bovine granulosa cells produce IGF-I. This assay was therefore used to accurately define gonadotrophic and environmental effects (nutrition) on peripheral IGF-I concentrations. Also to help confirm whether IGF-I is a major autocrine factor at the level of the ovaries.

4. EFFECTS OF DIETARY INTAKE ON THE RECRUITMENT OF OVARIAN FOLLICLES IN HEIFERS

4.1 ABSTRACT

The objective of this study was to determine whether dietary-induced changes in growth hormone (GH), insulin, and IGF-I altered the pattern of ovarian follicular development in heifers. Twenty-eight 2 to 3 years old Hereford-Friesian heifers were allocated equally ($n = 7$) to one of four dietary treatments: 1. control (C) fed on a maintenance diet as two meals per day. 2. twice maintenance (2M2) given as two meals per day; 3. Twice maintenance (2M6) given as six meals per day; and 4. Feed-deprived (F): Maintenance requirements as for C, changed to straw *ad libitum* for 3 days from a synchronised oestrus. On day 4 (oestrus = day 0) all heifers were transferred to grass silage (*ad libitum* access) until the end of the experiment. Blood samples were collected hourly for 10 h on day 1 and 3 and daily for a further 14 days.

Follicular development was monitored daily by ultrasonography until day 14. The number of small follicles (< 4 mm) was increased by 37% ($P < 0.05$) on day 1 and 2 in 2M2 and 2M6 heifers, with no carryover effect of nutrition to the second follicular wave. Numbers of medium-sized (4 to 8 mm) and large (> 8 mm) follicles did not vary ($P > 0.05$) between treatments. FSH concentrations were not different ($P > 0.05$) between treatments. Insulin concentrations were higher ($P < 0.05$) in 2M2 and 2M6 heifers than in C or F heifers, with no carryover after the end of treatment. The increase in number of small follicles was independent of changes in FSH and IGF-I, and was negatively correlated with GH, but positively associated with circulating insulin levels. These results indicate that a short-term increase in nutritional plane affects follicular recruitment in cycling heifers.

4.2 INTRODUCTION

The importance of nutrition in reproductive performance in farm animals is widely recognised (for reviews see: Dunn and Moss, 1992; Robinson, 1990; Wade and

Schneider, 1992). However, the mechanisms which mediate nutritional effects are poorly understood, and a number of putative mediators have been proposed.

Administration of recombinant GH was associated with an increase in both the number of small follicles (<5mm) in non-lactating heifers (Gong *et al.*, 1993a), and with a rise in both insulin and IGF-I. Although there is no direct evidence of an effect of IGF-I on follicular development in cattle, higher peripheral concentrations of IGF-I were associated with cattle selected for increased twinning rate (Echternkamp *et al.*, 1990), and IGF-I concentration in follicular fluid of oestrogen-active follicles was positively associated with follicular diameter (Spicer *et al.*, 1988). *In vitro*, GH (Gong *et al.*, 1994), insulin, and IGF-I (see Chapter 6 and 8) stimulate proliferation and steroidogenesis of bovine granulosa cells. Despite these associations, the specific roles of GH, IGF-I, and insulin on ovarian follicular development *in vivo* are not yet resolved.

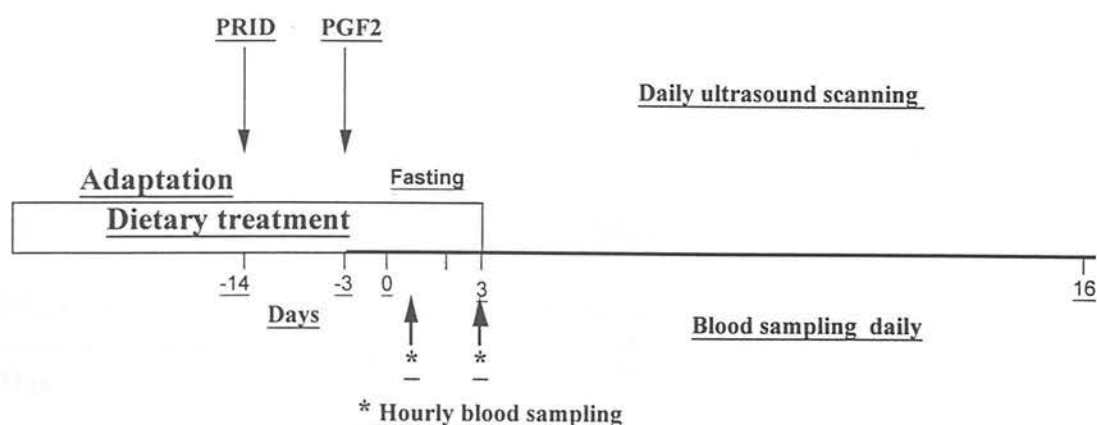
To study the influence of nutrition on follicular development, the quantity and frequency of dietary intake was altered to test the effects of dietary-induced alterations in physiological concentrations of peripheral GH, IGF-I, and insulin concentrations on follicular development.

4.3 MATERIALS AND METHODS

4.3.1 EXPERIMENTAL HEIFERS AND DIETARY TREATMENTS.

Twenty-eight 2 to 3 yr old Hereford-Friesian heifers, with an average body weight of 449 ± 6 kg, and body condition score 2 to 2.5, were penned together with wood shavings as bedding material. Heifers were randomly allocated to one of four dietary treatments and were offered food individually. The experimental protocol is outlined in Figure 4.1.

Figure 4.1. Experimental protocol



The compositions of the four diets, and the rations given are shown in Table 4.1. Dietary treatments were:

- 1) control heifers received 100 % maintenance requirements (C; n = 7) calculated according to AFRC (1993), given in two meals per day (10.00 and 16.30h).
- 2) received 200% maintenance requirements given in two meals per day (2M2; n = 7) at 10.00 and 16.30h. The increase in food intake was expected to decrease GH concentrations and increase insulin concentrations, but maintain IGF-I concentrations similar to those in C heifers.
- 3) received 200% maintenance requirements given in 6 meals (2M6; n = 7) per day (concentrate was given at 10.00, 11.30, 13.30, 15.00, 16.30, and 18.00, and hay was given at 10.00 and 16.30). As in 2M2, GH was expected to decline. Moreover, the increased frequency of feeding was designed to moderate the release of insulin, resulting in overall lower peripheral concentrations of insulin (Sutton *et al.*, 1986), and hence IGF-I.
- 4) Received a diet designed to provide 100% of the maintenance requirements, as for treatment 1, with a sudden change in diet to straw *ad libitum* (F; n = 7) for 3 days from day 0 (day 0 = oestrus) to day 3 of the synchronised oestrous cycle. Feed

restriction was expected to cause a rise in GH concomitant with a decline in plasma insulin and IGF-I concentrations.

Table 4.1. Daily allowances per dietary treatment and composition of food ingredients

Daily allowances			
Treatment	100%	200%	Feed deprived
(Kg DM per day)	(control)	(2x ¹ and 6x ²)	
Hay	2	2	--
Concentrate	2.5	6.5	--
Straw	--	--	4.6

Chemical Analysis of Feed

	Hay	Straw	Concentrate
DM g per Kg	913	901	863
per Kg DM:			
ME (MJ)	8.3	4.3	12.8
CP (g)	83	44	227
OM	942	961	927
ADF	288	540	148
NDF	664	835	220

¹2x heifers fed twice daily

²6x heifers fed six times a day

For 2 weeks before the experiment, heifers were halter-trained whilst adapting to their diet. All heifers were consuming the whole dietary treatments for 2 weeks before synchronised oestrus, and remained on the appropriate diet until day 3 after oestrus.

From day 4 of the oestrous cycle, heifers were penned together and offered perennial rye-grass silage *ad libitum* for the rest of the study. Food intake was not measured. Body weight was recorded at the beginning of the adaptation period, and once a week until 3 days after the end of the dietary treatments. Oestrus was synchronised and frequent blood samples were collected at 0 (before the morning feed), 30 and 60 min after feeding and every hour for 10 h on day 1 and 3 of the synchronised oestrous cycle. Daily blood samples were then collected from day 4 until day 14 of the synchronised oestrous cycle, and the pattern of follicular development was recorded daily from day 1 to 14 of the synchronised oestrous cycle.

4.3.2 HORMONE AND METABOLITE ANALYSIS.

Plasma samples were analysed in single assays for FSH (intra-assay coefficient of variation (CV)= 1.2%), insulin (CV= 7.4%), GH (CV= 8%), progesterone (CV= 3.9%), IGF-I (CV= 3.8%), urea (CV= 1.8%), glucose (CV= 1.6%) and BOHB (CV= 4.3%).

4.3.3 STATISTICAL ANALYSES.

Differences in body weight change between treatments were analysed using Student's paired *t*-test. The effect of dietary treatment on the time course of the response in insulin, GH, FSH, and metabolites on pooled data from the serial samples (day 1 and 3) was compared by homogeneity of regression. Variation in mean concentrations of hormones or metabolites and in the number of follicles between treatments, during the treatment period and during the first 14 days of the oestrous cycle were analysed by split-plot analysis with repeated measurements, allowing for variation of treatment, animal within treatment, day of the oestrous cycle and the interaction of day X treatment. The significance of the effect of dietary treatment was tested using the animal within treatment as an error term.

4.4 RESULTS

4.4.1 ANIMAL BODY WEIGHT.

At the start of the experiment, there was no difference in the average body weight among treatments. By three days after the end of the nutritional treatment, C heifers had not gained weight (4 ± 4 kg; $P > 0.05$), whereas heifers in treatments 2M2, 2M6, and F were (23 ± 7 , 30 ± 5 , and 9 ± 1 kg respectively) heavier ($P < 0.01$) than at the beginning of the experiment. However, the weight gain in F heifers did not differ ($P > 0.05$) from that of C heifers.

4.4.2 ACUTE EFFECTS OF DIET ON HORMONE AND METABOLITE CONCENTRATIONS.

Regression analysis of pooled data from serial blood samples (day 1 and 3) showed three distinct responses to nutrition. In heifers fed straw (F) for 3 days after oestrus (Figure 4.2a), insulin concentrations did not change ($P > 0.05$) during the sampling period. These heifers had the lowest ($P < 0.01$) mean concentration of insulin (0.27 ng/mL) of all the treatments. In the other 3 treatments, apart from having higher ($P < 0.05$) basal insulin levels, insulin concentrations increased ($P < 0.01$) in response to feed intake according to the diet (Figure 4.2a). Basal insulin concentrations in control heifers were 0.4 ± 0.03 ng/mL. Insulin increased ($P < 0.01$) with time in response to feed intake, and this response was described by the regression equation $y = 0.4 + 0.023x$ ($R^2 = 0.53$; $P < 0.01$). Heifers in the 2M2 and 6M6 treatments had greater ($P < 0.01$) basal insulin concentration (0.56 ± 0.03 ng/mL) than C heifers. The insulin response to twice- or six-times daily feeding was described in both treatments by the regression equation $y = 0.56 + 0.97x$ ($R^2 = 0.53$; $P < 0.01$).

As with insulin, 3β -hydroxybutyrate (BOHB, Figure 4.2b) increased with time ($P < 0.01$) in direct relation to feed intake. However, there was no difference ($P > 0.05$) in the response of BOHB over time between straw- and C-fed heifers (pooled regression equation $y = 0.2 + 0.005x$; $R^2 = 0.57$), or between 2M2- and 2M6-fed heifers ($y = 0.2 + 0.079x - 0.005x^2$; $R^2 = 0.57$). Urea concentrations (Figure 4.2c) between F- and C-fed heifers differed ($P < 0.01$) in magnitude, (F: $y = 2.96 + 0.1x$; C: $y = 4.08 + 0.1x$; $R^2 = 0.73$), whereas in 2M2 and 2M6 heifers, urea concentrations differed ($P < 0.01$) in both magnitude and over time ($y = 5.8 + 0.18x - 0.02x^2$; $R^2 = 0.73$) from C and F

heifers, with no difference ($P > 0.05$) between 2M2- and 2M6-fed heifers. Mean glucose concentrations (Figure 4.2d) did not vary ($P > 0.05$) between treatments, or with time after feeding ($P > 0.05$).

Growth hormone and FSH concentrations with time after feeding did not differ ($P > 0.05$) between dietary treatments.

As there were no differences in metabolite or insulin concentrations in heifers consuming 200% maintenance requirements with different frequencies of feeding (2M2 and 2M6), these were considered as one group (2M) for further analysis.

4.4.3 EFFECT OF DIET ON FOLLICULAR DEVELOPMENT.

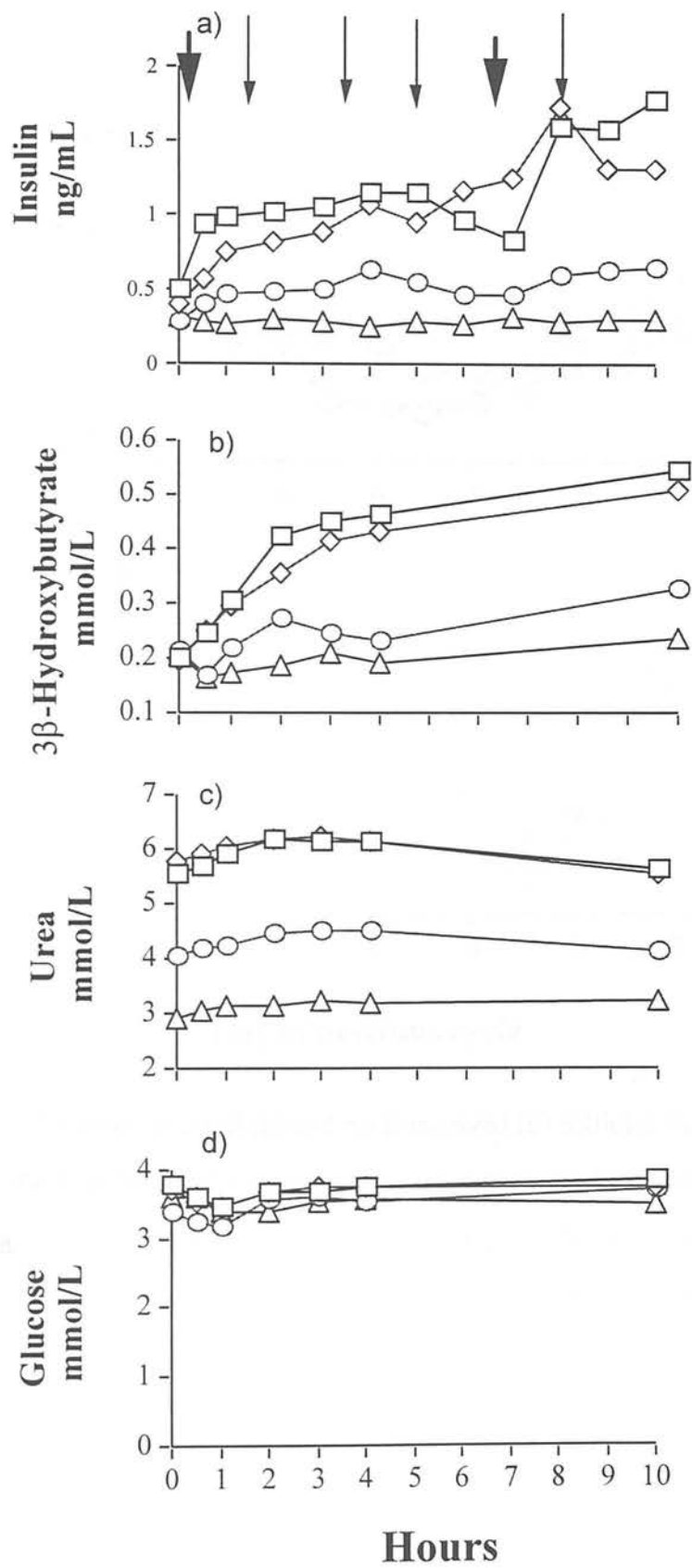
The number of small follicles was increased by 37% ($P < 0.05$) on day 1 and 2 of the oestrous cycle in 2M heifers (Figure 4.3a) compared to C and F heifers. There was an effect ($P < 0.01$) of day of oestrous cycle on the number of small follicles. No differences ($P > 0.05$) were observed after day 3 of the oestrous cycle in the number of small follicles between treatments. Number of small and medium-sized follicles varied ($P < 0.01$) with day of the oestrous cycle. The highest number of small follicles was seen on day 2 and 12 (Figure 4.3a), and for medium-size follicles (Figure 4.3b) on day 4 and 14 of the oestrous cycle. Medium-sized (Figure 4.3b) and large (data not shown) follicle numbers were unaltered by dietary treatments ($P > 0.05$).

4.4.4 EFFECT OF DIET ON HORMONE AND METABOLITE CONCENTRATIONS DURING THE OESTROUS CYCLE.

Follicle-stimulating hormone concentrations (Figure 4.4a) did not vary ($P > 0.05$) between treatments. However, FSH concentrations did vary ($P < 0.05$) with day of the oestrous cycle. Follicle-stimulating hormone levels peaked on day 1 and 9 of the oestrous cycle, preceding the peak in number of small follicles.

Progesterone concentrations increased ($P < 0.01$) with day of the oestrous cycle (Figure 4.4b), and heifers on the high plane of nutrition had higher (day by treatment interaction; $P < 0.05$) progesterone concentrations on day 10 to 12 of the cycle than C and F heifers.

Figure 4.2. Time course responses of insulin (a), 3 β -hydroxybutyrate (b), urea (c), and glucose (d) to feeding in control (C, \bigcirc), feed restricted (F, \triangle), and 200% maintenance diets given in 2 (2M2, \square) or 6 (2M6, \diamond) rations daily. Blood samples were taken at 0, 0.5, 1, and every hour for 10h. Food was administered at 0 and 6.5h (short arrows) for heifers eating twice daily, and at 0, 1.5, 3.5, 5, 6.5, and 8h for group 2M6 (long arrows). SEM values were 0.09 for insulin, 0.18 for 3 β -hydroxybutyrate, 0.13 for urea and 0.06 for glucose.



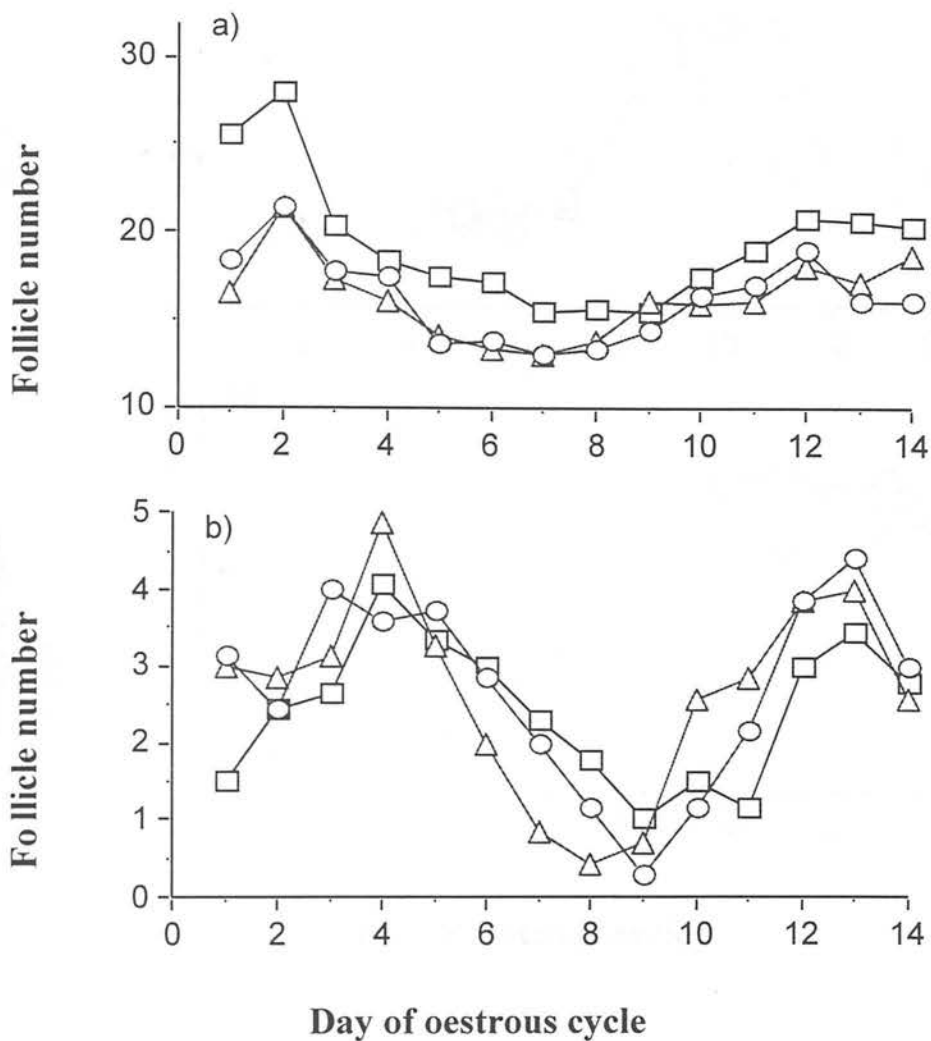


Figure 4.3. Number of small (a) and medium-sized (b) follicles from day 1 to 14 of the synchronised oestrous cycle (oestrus = day 0) in control (C, \bigcirc), feed restricted (F, \triangle), and 200% maintenance (2M, \square) treatments. Dietary treatment was given until day 3 of the oestrous cycle, and there was no control over food intake thereafter. SEM values were 2.4 (group C and F; $n = 7$) and 1.7 (group 2M; $n = 14$) for small, and 0.77 (group C and F; $n = 7$) and 0.5 (group 2M; $n = 14$) for medium-sized follicles.

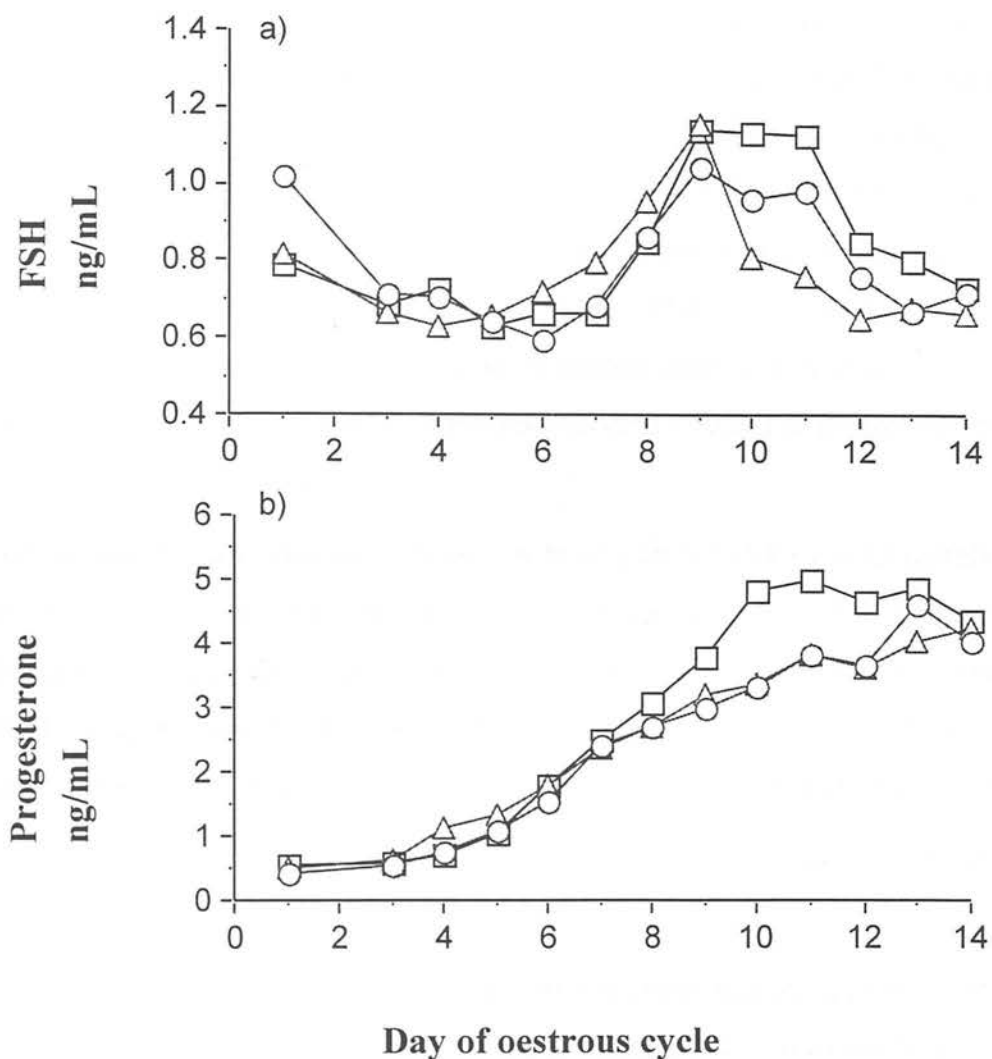


Figure 4.4. FSH (a) and progesterone (b) concentrations from day 1 to 14 of the oestrous cycle (oestrus = day 0) in control (C, \bigcirc), feed restricted (F, \triangle), and 200% maintenance (2M, \square) treatments. Dietary treatment was given until day 3 of the oestrous cycle, and there was no control over food intake thereafter. S.E.M values were 0.08 (group C and F; $n = 7$) and 0.06 (group 2M; $n = 14$) for FSH, and 0.25 (group C and F; $n = 7$) and 0.18 (group 2M; $n = 14$) for progesterone concentration.

During the first three days of the synchronised oestrous cycle, insulin concentrations differed ($P < 0.05$) between treatments (Figure 4.5a). Heifers on the 2M treatment diet had higher insulin ($P < 0.01$) concentrations (1.08 ± 0.04 ng/mL) than heifers on the C treatment (0.51 ± 0.07 ng/ml), and these in turn had higher ($P < 0.01$) insulin levels to heifers in treatment F (0.29 ± 0.06 ng/ml). After withdrawal of nutritional treatments, insulin concentrations in heifers on the 2M diet decreased by day 3 to concentrations similar ($P > 0.05$) to those of control heifers, and insulin levels in heifers in group F increased to concentrations similar ($P > 0.05$) to control heifers on day 5.

Growth hormone concentrations in heifers in treatments F (15.86 ± 1.12 ng/ml) and C (16.59 ± 1.12 ng/ml) did not vary ($P > 0.05$) during the first 3 days of the synchronised oestrous cycle. However, 2M heifers had lower GH concentrations (10.9 ± 0.79 ng/ml) than C and F heifers ($P < 0.05$), but GH concentrations increased to concentrations similar to those in C heifers from day 4 until the end of the study.

Total IGF-I (Figure 4.5c) concentrations did not vary either between treatments, or with day of the oestrous cycle ($P > 0.05$).

During the first 3 days of the synchronised oestrous cycle, heifers in groups 2M had higher ($P < 0.05$) BOHB (Figure 4.6a) and urea (Figure 4.6b) concentrations ($.38 \pm 0.007$ and 5.95 ± 0.09 mmol/L) than C heifers ($.24 \pm 0.01$ and 4.34 ± 0.13 mmol/L, respectively). Urea concentrations in heifers in group F differed ($P < 0.05$) from C heifers only after 3 days of feed restriction (3.16 ± 0.13), and this difference was maintained until 4 days after the end of treatment. Glucose concentrations did not differ ($P > 0.05$) between treatments or with day of the synchronised oestrous cycle (Figure 6c).

Figure 4.5. Insulin (a), GH (b), and IGF-I (c) concentrations from day 1 to 14 of the oestrous cycle (oestrus = day 0) in control (C, ○), feed restricted (F, △), and 200% maintenance (2M, □) treatments. Dietary treatment was given until day 3 of the oestrous cycle, and there was no control over food intake thereafter. SEM values were 0.09 (group C and F; n = 7) and 0.06 (group 2M; n = 14) for insulin; 2.3 (group C and F; n = 7) and 1.6 (group 2M; n = 14) for GH and 39.6 (group C and F; n = 7) and 28 (group 2M; n = 14) for IGF-I.

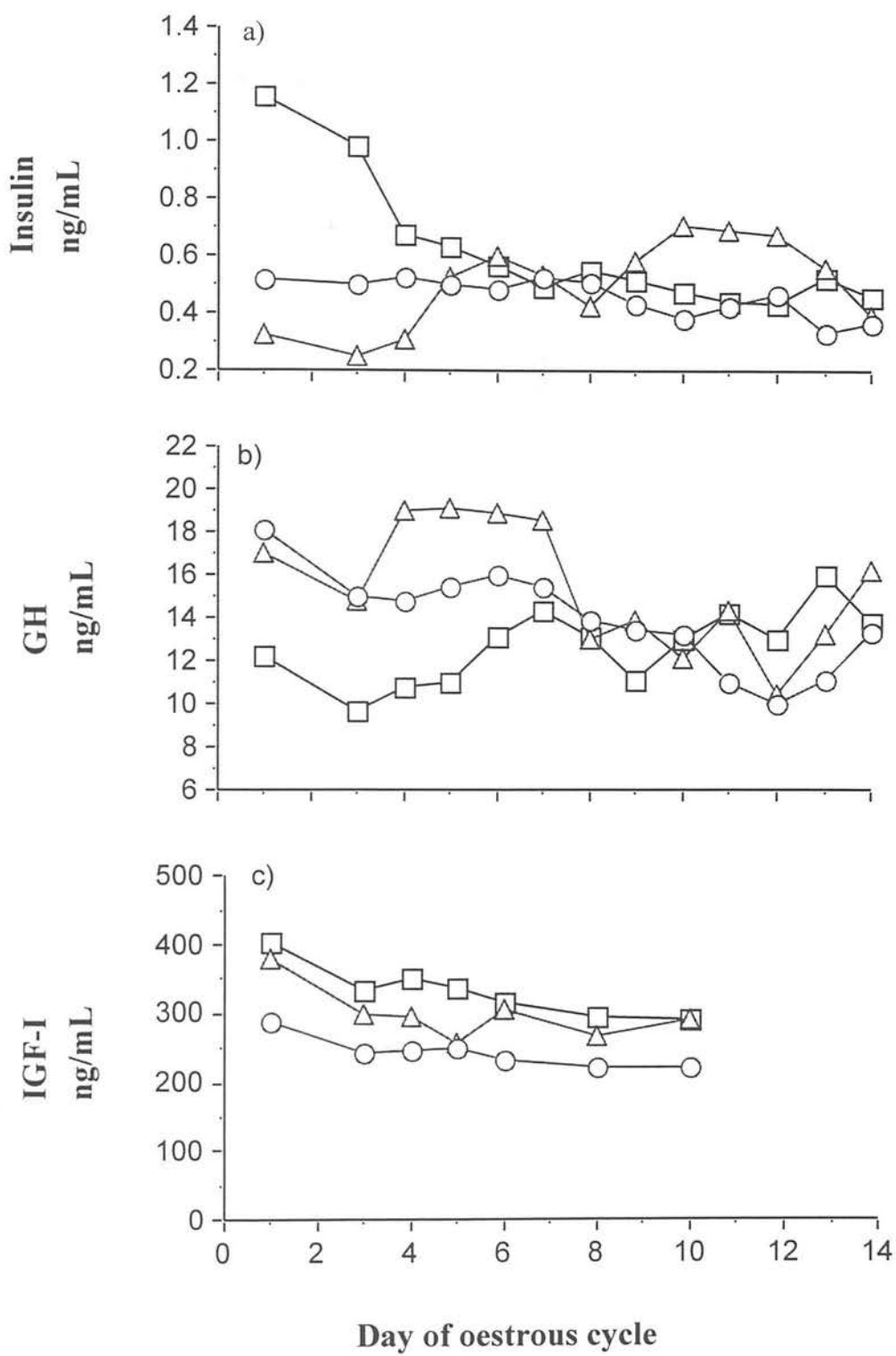
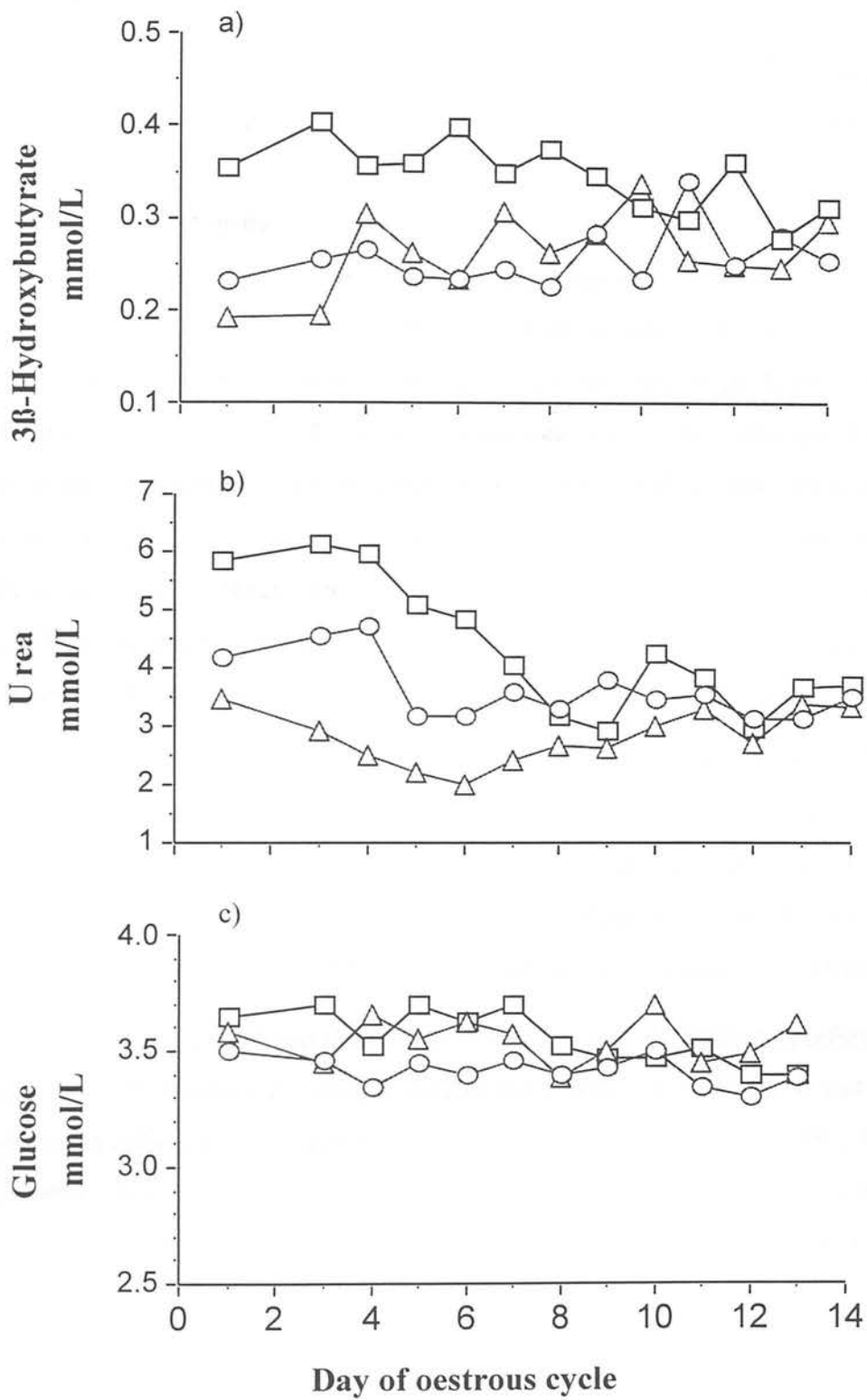


Figure 4.6. 3β -Hydroxybutyrate (a), urea (b), and glucose (c) concentrations from day 1 to 14 of the oestrous cycle (oestrus = day 0) in control (C, \bigcirc), feed restricted (F, \triangle), and 200% maintenance (2M, \square) treatments. Dietary treatment was given until day 3 of the cycle, and there was no control over food intake thereafter. SEM values were 0.04 (group C and F; $n = 7$) and 0.02 (group 2M; $n = 14$) for 3β -hydroxybutyrate, for urea 0.28 (group C and F; $n = 7$) and 0.2 (group 2M; $n = 14$), and for glucose 0.1 (group C and F; $n = 7$) and 0.07 (group 2M; $n = 14$).



4.5 DISCUSSION

Increased dietary intake was associated with a significant increase in small follicle recruitment during the first follicular wave of the oestrous cycle in Hereford-Holstein heifers. This increase was lost rapidly as soon as dietary treatments terminated. Numbers of medium-sized follicles increased in all treatments, coincident with the decrease in the numbers of small follicles. Taken together, these results indicate that nutrition appears to affect the recruitment of small follicles, but not follicle selection and dominance. Similar observations were made by Gong *et al.* (1993a) following GH treatment *in vivo*. In an early study, Maurasse *et al.* (1985) reported that altering the plane of nutrition induced changes in ovarian follicle size distribution and increased follicle numbers in cattle. In sheep, a period of improved nutrition (flushing) increases the number of follicles present in the ovary and ovulation rate (see Downing and Scaramuzzi, 1991). In cattle, this is the first time that small follicle recruitment has been shown to be affected by nutritional treatment around oestrus.

Undernourishment is also known to affect the growth of the dominant follicle in cattle (Murphy *et al.*, 1991). Long-term periods of undernutrition induce anoestrus and decreased the number of small (<6mm) follicles recruited (Gutierrez *et al.*, 1994). However, in the present study, short-term feed restriction did not affect the number of small follicles, confirming the observations of Spicer *et al.* (1992b).

The mechanisms underlying the effects of nutrition on reproductive performance are still not well understood. Several studies have failed to establish a link between nutritional effects and changes in gonadotrophins (Roberson *et al.*, 1992; Rhodes *et al.*, 1995). In this study, as in others (Gong *et al.*, 1995; Adams *et al.*, 1994), FSH concentrations varied with day of the oestrous cycle in a wave-like pattern, in phase with the numbers of small follicles. No differences in FSH concentrations, or in FSH secretion pattern, were observed between different nutritional treatments. Therefore, changes in the number of small follicles, induced by increased nutrient intake, during the first 3 days of the oestrous cycle, seem to be independent of changes in circulating FSH concentrations.

Growth hormone, IGF-I, and insulin are physiologically inter-related and respond to nutritional changes (Pell and Bates, 1990; Thissen *et al.*, 1994), and are proposed as putative mediators between nutrition and reproduction. Concentrations of insulin and IGF-I increase with nutrient intake, whereas GH concentrations decrease as nutrient intake decreases (Foster *et al.*, 1989; Clarke *et al.*, 1993; Breier *et al.*, 1986). In the present study, insulin concentrations increased in heifers on the high dietary intakes, and this was associated with an increase in the number of small follicles during the first 3 days of the oestrous cycle. The increase in insulin seen in heifers on the high plane of nutrition was associated with an increase in the number of small follicles. *In vitro*, insulin stimulates proliferation and steroidogenesis of both bovine (see Chapter 6) and ovine granulosa cells (Campbell *et al.*, 1996a). In pigs, exogenous insulin increased the number of small follicles (<3mm) and reduced the number of atretic follicles (Matamoros *et al.*, 1990; 1991). However in heifers, exogenous insulin *in vivo* failed to induce a stimulation of follicular development (Simpson *et al.*, 1994). Therefore, insulin may be one of the mediators of the effects of nutrition on follicular development.

Small follicle numbers did not differ between F and C heifers, although insulin concentrations in F heifers were lower (Figure 4.5a), but only after 3 days of feed restriction. Changes in the small follicle population have been shown to occur approximately 48 h after changes in insulin and IGF-I following GH treatment *in vivo* (Gong *et al.*, 1993a). Hence, it is probable that the decrease in insulin was either too late and/or too small to effect changes in follicle development.

The lack of difference in IGF-I concentrations between treatments indicates that extra-ovarian IGF-I does not account for the observed changes in follicular development. The IGF-I concentrations reported in this study are relatively high compared with concentrations reported previously for cattle in similar physiological conditions (Armstrong *et al.*, 1993; Spicer *et al.*, 1992b). It is likely that these differences are due to the different extraction protocols used for the apparent removal of IGF-binding proteins (IGFBPs). As shown earlier (see Chapter 3), results can vary depending on the IGF-I/IGFBPs ratio, underestimating true IGF-I values. This effect

is accentuated even further at higher IGF-I concentrations and IGFBPs remaining after extraction can interfere in the assay, giving inaccurate results, unless acid chromatography is used (see Chapter 3). This was the case in the present study. Unfortunately, as far as we are aware, there are no other available reports of bovine IGF-I measurements using acid gel chromatography for comparison.

In cattle, it is still not established whether IGF-I is synthesised by granulosa cells. We have been unable to demonstrate the presence of IGF-I or its mRNA in bovine granulosa cells either before or after culture (see Chapter 3 and 10). In addition, IGFBPs (Armstrong *et al.*, 1996c) and IGFBP-proteases (Besnard *et al.*, 1996) are both produced within the follicle. Thus, the bioavailability of IGF-I probably changes markedly at the follicular level. It is likely therefore that, in addition to effects of insulin, extra-ovarian sources of IGF-I enhance follicular growth and steroidogenesis (Campbell *et al.*, 1995), and may account for some of the pharmacological effects of BST (Gong *et al.*, 1993a), and some of the physiological effects of nutrition on follicular development seen in the present study. More research addressing the role of IGFBPs in the regulation of IGF-I action on follicular development is necessary to clarify these issues, particularly since nutritional status can alter both the quantity and species of the IGFBPs in the peripheral circulation (McGuire *et al.*, 1992).

Growth hormone is unlikely to be involved in the increment in small follicle numbers seen in this study, as the number of small follicles in 2M heifers increased when there was a decline in GH concentration (Figure 4.5b). Although GH has been associated with increases in follicle number (Gong *et al.*, 1993a; De La Sota *et al.*, 1993), there is evidence that ovarian follicles do not possess GH receptors (Lucy *et al.*, 1993a). In addition, if heifers are fed above their nutritional requirements, GH decreases, while IGF-I and insulin increase (Clarke *et al.*, 1993; Breier *et al.*, 1986; Thissen *et al.*, 1994). Growth hormone and IGF-I levels in F heifers did not change, possibly because the period of nutritional deprivation was not sufficiently long. This is supported by the observation that insulin concentrations in heifers in group F, although lower, only differed from controls after 3 days of feed restriction (Figure 4.5a). Similar results on GH concentrations after a short fasting period were observed

by Ward *et al.* (1992). Therefore, the effects of GH administration on follicular development are most likely to be mediated by changes either in insulin and/or IGF-I.

In heifers on the high level of nutrition, progesterone concentrations rose faster than in controls (Figure 4.4b), and were greater on day 10 to 12 of the oestrous cycle. Progesterone has been found to have a negative relationship with nutrition (Dunn and Moss, 1992). However, others found a positive relationship (Richards *et al.*, 1995). These results indicate that the level of nutrition during the development of the ovulatory follicle or early formation of the corpus luteum may affect the further luteal development.

3 β -hydroxybutyrate concentrations are an indicator of energy balance, with high values indicating a negative energy balance (Perotti *et al.*, 1989). 3 β -hydroxybutyrate is produced from the partial oxidation of free fatty acids mobilised from body fat reserves by the liver in periods of undernutrition. However, in ruminants, BOHB is also produced in the ruminal wall from butyric acid, which is a product of rumen fermentation. In this study, the high BOHB concentrations in 2M heifers was probably from ruminal rather than hepatic origin, because the concentrations of BOHB related positively with food intake (Figure 4.2b; Vernon and Peaker, 1983). The period of feed restriction failed to produce the desired effect, since BOHB in F heifers did not differ from C heifers. Our results showed no difference in glucose concentrations with diet or time after feeding. Ruminants use volatile fatty acids as major sources of energy, and changes between the post-absorptive and starving states are low and rapidly regulated by insulin. Urea concentration is an indicator of protein consumption (Calamari *et al.*, 1989), and as expected, had a positive relationship with diet. Evaluation of the dietary regimens adopted in this experiment highlights the inherent difficulties of studies on the effects of acute feed restriction in ruminants. Contrary to the situation in non-ruminant animals, the rumen can supply nutrients for an extended period of time. The increase in the proportion of concentrate to fibre in the 2M treatment favoured a quick release of carbohydrates, provoking an increase in insulin (Sutton *et al.*, 1986). However, greater frequency of

feeding (6 instead of 2) did not change the pattern of insulin secretion in contrast to what was expected. Sutton *et al.* (1986) found that 6 meals spread over a 24 h period reduced the amplitude of the insulin response to feeding, provoking a reduction in the overall insulin concentration compared to twice-daily feeding. The effect was not observed, perhaps because the distinction between 2M2 and 2M6 feeding was moderated by the overall lower plane of feeding in this study with heifers compared to that of Sutton *et al.* (1986) in lactating cows.

In conclusion, our results demonstrate that nutrition can stimulate changes in the number of small follicles which are independent of changes in peripheral FSH levels, but positively associated with circulating insulin concentrations. GH and total IGF-I concentrations were not related to the increase in small follicle recruitment.

Development of a practical procedure or pharmacological treatment that would increase the pool of follicles available for oocyte collection or superovulatory treatments would undoubtedly benefit the embryo transfer industry. Although this study has clearly shown that increased dietary intake can acutely enhance the recruitment of small follicles, further work is required to determine whether this would result in improved superovulatory responses. This study has demonstrated that insulin, but not FSH or GH, is the most likely hormone to be involved. However the roles of both extra- and intra-ovarian IGF-I and IGFBPs need to be examined further.

5. EFFECT OF GENETIC SELECTION FOR MILK YIELD ON METABOLIC HORMONES AND FOLLICULAR DEVELOPMENT IN POSTPARTUM DAIRY CATTLE

5.1 ABSTRACT.

The relationship between endocrine and metabolic changes associated with genetic selection for predicted breeding value (PBV) for milk yield with follicular development postpartum was investigated. PBV was strongly correlated with milk production during the first 305 days of lactation. High PBV was associated with higher GH and BOHB, and lower insulin and glucose concentrations. Although no differences were observed in follicular development between selection lines, changes in body weight appeared to influence the number of follicles present in the ovary. Furthermore, the day to first ovulation postpartum was delayed in cows selected for high milk-yield.

5.2 INTRODUCTION

The gain in productive efficiency in dairy cattle achieved in the last decades has undermined reproductive efficiency (for reviews see Butler and Smith, 1989; Swanson, 1989; Bauman, 1992). High milk yields in dairy cattle are positively associated with GH concentrations (Barnes *et al.*, 1985). In addition, exogenous GH administration enhances milk production (Peel and Bauman, 1987) and provokes metabolic responses similar to those caused by genetic selection for higher milk production (Bauman, 1992; Kazmer *et al.*, 1986). Nowadays, recombinant GH administration is widely used to increase milk production in North America and was associated with increased twinning rate (Cole *et al.*, 1991).

The above observations encouraged investigators to study the effects of GH administration on follicular development in cattle. In beef heifers, Gong *et al.* (1991)

found that GH administration increased small follicle (<5mm) numbers, whereas an increased number of medium-sized (5-10mm) follicles was seen in lactating animals (De la Sota *et al.*, 1993; Lucy *et al.*, 1993b). In the last Chapter, the effect of increased dietary intake on stimulating the number of small follicles was demonstrated. However, short-term feed restriction did not affect follicle number in any size category. In this study, the metabolic environment associated with selection for high or low genetic merit for milk production and the relationship between genetic-associated endocrine milieu and follicular development in the postpartum period was investigated.

5.3 MATERIALS AND METHODS

5.3.1 HERD MANAGEMENT

The study was conducted on the Institute's farm (Blythbank, Tweeddale, Scotland). The herd is a commercially managed Holstein-Friesian dairy herd that has been differentially selected for either high (H) or low (L) PBV for milk yield (difference between lines =1400 kg milk in 305 days of lactation). Animals were kept inside sheds during the winter months (October to April), whilst during the summer (May to September) they were kept on pasture.

5.3.2 DIETS

After calving, cows were fed a ration consisting of perennial rye grass silage (36 kg) and concentrate (6 kg; Dairy Pellets; Dalgety Agriculture Ltd.). Dietary intakes were calculated to provide maintenance and lactation requirements for 24 litres (Lt) of milk production (Table 5.1). In the milking parlour, cows received an extra 0.4 kg of concentrate for every litre produced above the 24 Lt provided in the diet. Heifers were supplemented with one kilogram extra of concentrate (Gold 19 nuts; Dalgety) for growing requirements. After milking, animals were given *ad libitum* access to grass silage for one hour, and then released to the field where they remained between milking sessions. Milking was carried out twice daily (05.00 and 14.30 hours).

Table 5.1. Daily food intake and adequacy of nutrients relative to daily nutritional requirements for maintenance and milk production.

	Intake	Adequacy (%)
DM	16.5 Kg	
ME	193 MJ	101
CP	1.66 Kg	107
ERDP:FME	12.2 g/MJ	113
MADF	22.1 % DM	
Oil	5.5 % DM	

DM = dry matter; ME = metabolisable energy; CP = crude protein; ERDP = effective rumen degradable protein; FME = fermentable metabolisable energy; MADF = modified acid detergent fibre

5.3.3 EXPERIMENTAL ANIMALS

Thirty-six Holstein-Friesian cows, calving between April 26 and May 28, 1995, from either H (n=18) or L (n=18) milk yield lines were studied from day 15 to 35 postpartum, when a preliminary pilot study indicated that the first ovulation postpartum occurred.

Every day, before the afternoon milking session, blood samples (10 ml) were collected and follicular development examined using an ultrasound scanner as described in Chapter 2. Follicles were classified according to their diameter as small (<4mm), medium-sized (4-8mm) or large (>8mm) follicles. Ovulation was identified by the disappearance of an identified dominant follicle. Plasma FSH (intra-assay CV= 3%), progesterone (CV= 8.1%), insulin (CV= 4.3%), GH (CV= 3.9%) and BOHB, urea and glucose were measured in a single assay.

5.3.4 BODY WEIGHT CHANGES

Body weight was recorded weekly throughout the duration of the experiment. Daily weight change between weekly measurements was calculated by the difference

between consecutive weight measurements. The values were subjected to third-order regression analysis (Lucy *et al.*, 1991a) to obtain a predicted change in body weight (PCBW). The linear and quadratic terms of the regression equation were statistically significant ($R^2 = 0.37$) and the PCBW for each animal at each day of the study were used as covariates for further analysis.

5.3.5 STATISTICAL ANALYSES

Statistical analysis was performed by general linear models procedure. Milk production in a 305 days lactation was analysed in a model containing genetic line and parity (heifer or cow), and the effects of PBV for milk yield on milk production in 305 days determined by regression analysis. Milk production during the period of study was analysed in a model containing genetic line, cow nested within line, parity and the interactions between parity, line and day postpartum. Numbers of follicles within each size category and FSH concentrations were analysed in a similar model as described above including (1) days postpartum (dpp), (2) presence or absence of a dominant follicle or (3) day relative to first ovulation postpartum. The effect of the PCBW was analysed by including it as a covariate in the analysis. The effect of genetic line was tested with cows nested within lines as the error term.

The differences in the day to first ovulation postpartum were analysed by Student's *t*-test. Cows which did not ovulate during the study were assigned an arbitrary value of 35 days. Differences in the proportion of cows ovulating during the study in the two genetic lines was tested by Fisher's exact test. The effect of line on mean concentrations of metabolic hormones and metabolites was tested as for milk production and by regression analysis, testing for homogeneity of slopes between lines. Stepwise regression analysis was used to determine which metabolic hormones and metabolites were associated with PCBW. Finally, Pearson correlation coefficients were calculated for the relation between GH, insulin and metabolites with number of follicles in each size category. Significant differences were declared at $P > 0.10$.

5.4 RESULTS

5.4.1 MILK PRODUCTION

As expected, average milk production in 305 days of lactation differed ($P < 0.01$) between lines. Cows selected for high milk yield produced more milk (6880 ± 164.3 kg) than cows selected for low (5795.3 ± 317.4 kg) milk yield. Predicted breeding value for milk production was strongly positively correlated with milk yield during the 305 days of lactation (regression coefficient = 1.08). During the period of study, average daily milk production did not differ ($P > 0.10$) between animals of H (30.96 ± 1.51 kg/day) or L (30.17 ± 1.72 kg/day) genetic merit. However, animals of first lactation (heifers) produced less ($P < 0.01$) milk than those of second or more lactations. For both genetic lines, milk production increased ($P < 0.01$) with day postpartum ($Y = 21.18 + 1 \text{ dpp} - 0.018 \text{ dpp}^2 - 12.41$ heifers; $R^2 = 0.64$), with no difference between lines ($P > 0.10$).

5.4.2 FOLLICULAR DEVELOPMENT

Four cows (3 L and 1 H) developed follicular cysts during the study and were excluded from further analysis. At day 15 postpartum, all cows had active ovaries containing follicles of all three size categories. The mean number of follicles within each class category (Figure 5.1), and the total number of follicles between days 15 to 35 postpartum did not vary ($P > 0.10$) between lines. However, small ($P < 0.07$), medium-sized ($P < 0.01$) and total ($P < 0.01$) number of follicles increased with days postpartum. The inclusion of PCBW as a covariate in the analysis, increased the probability associated with days postpartum for small ($P = 0.63$) and medium-sized ($P = 0.046$) follicle numbers, indicating that changes in number of small and medium-sized follicles, with increasing days postpartum, were associated with changes in body weight.

When follicle numbers and FSH concentrations were analysed according to the presence or absence of a dominant follicle (Table 5.2), the presence of the dominant follicle significantly reduced ($P < 0.01$) medium-sized and total follicle numbers and

FSH concentrations. In contrast, small follicle numbers were not affected ($P>0.10$) by the presence of the dominant follicle. However, when PCBW was included as a covariate in the analysis, the presence of the dominant follicle tended to decrease ($P=0.09$) small follicle numbers. Genetic line did not ($P>0.10$) affect the number of follicles in any of the three size categories. However, there was a low but significant interaction between line and the presence of a dominant follicle for small ($P=0.07$) and total ($P=0.07$) follicle numbers.

Table 5.2. Effect of the presence (1) or absence (0) of the dominant follicle (Dom) on small (<4mm), medium-sized (4-8mm), large (>8mm) and total follicle numbers and on mean FSH concentrations. Values are least square means \pm SEM. a, b indicate significant differences ($p<0.01$) within the same follicle-size category.

	Dom	small	medium	Large	Total	FSH
0		14.7 \pm 0.3	5.0 \pm 0.2 ^a	0.94 \pm 0.05	20.6 \pm 0.3 ^a	1.05 \pm 0.02 ^a
1		14.1 \pm 0.2	3.3 \pm 0.1 ^b	1.5 \pm 0.03	18.9 \pm 0.2 ^b	0.87 \pm 0.02 ^b

First ovulation postpartum occurred at 20.2 ± 1.6 days for cows selected for low milk-yield compared to 27.23 ± 1.9 days for high milk-yielders. The proportion of L cows (15/15) ovulating during the study was higher ($P<0.05$) than for H cows (12/17). However, when the cystic animals were included in this analysis the difference was no longer significant ($P>0.10$). Analysis of follicle numbers relative to day of ovulation showed that follicle numbers in all three size categories and FSH concentrations ($P<0.01$) varied with day (Figure 5.2). However, there were no differences between lines in number of follicles (day by line interaction; $p>0.10$) before ovulation. After ovulation, both follicle numbers for all follicle size categories and FSH concentrations varied with day ($P<0.01$), with no difference between lines observed.

5.4.3 BODY WEIGHT CHANGES

Average body weight change between the first and second weight measurements was negative (-1.3 ± 0.3 kg). As lactation advanced, average body weight change became progressively positive, and in the last week of study body weight change was 0.56 ± 0.21 kg. Ovulation occurred when the PCBW became positive (0.09 ± 0.2 kg), with no significant differences between lines ($P > 0.10$).

5.4.4 METABOLIC HORMONES AND SERUM METABOLITE CONCENTRATIONS

Table 5.3 shows mean serum concentrations for metabolic hormones and metabolites during the study period. GH and BOHB concentrations were higher ($P < 0.01$) in H than L cows. In contrast, insulin ($P < 0.06$) and glucose ($P < 0.05$) concentrations were lower in H than L cows.

Concentrations of GH and BOHB decreased ($P < 0.01$) with day postpartum. However, the rate of decline did not vary ($P > 0.10$) between lines, and serum concentrations were always higher in H cows than L cows. Mean glucose concentrations also differed ($P < 0.01$) between lines, being higher for L than H cows. There was an increase ($P < 0.01$) in glucose concentrations with day postpartum that was similar for both genetic lines ($P > 0.10$). Insulin concentrations differed between lines, but no changes were seen due to day postpartum ($P > 0.10$). Mean urea concentrations did not differ between H and L cows. However, while urea concentrations did not vary ($P > 0.10$) in H cows with day postpartum, in L cows, urea concentrations declined ($P < 0.01$) as lactation progressed.

The metabolic indices that best correlated (analysed by stepwise regression) with PCBW were BOHB, urea and GH (regression $y = -0.67 x_1 + 0.17 x_2 - 0.03 x_3$; where $y = \text{PCBW}$, $x_1 = \text{BOHB}$, $x_2 = \text{urea}$ and $x_3 = \text{GH}$; $R^2 = 0.17$).

Figure 5.1. Mean numbers of small (a), medium-sized (b), large (c) and total follicle numbers (d) observed by ultrasonography between days 15 to 35 postpartum. SEM values were 1.23, 0.89, 0.15 and 2.06 for small, medium-sized, large and total follicle number respectively.

Follicle Number

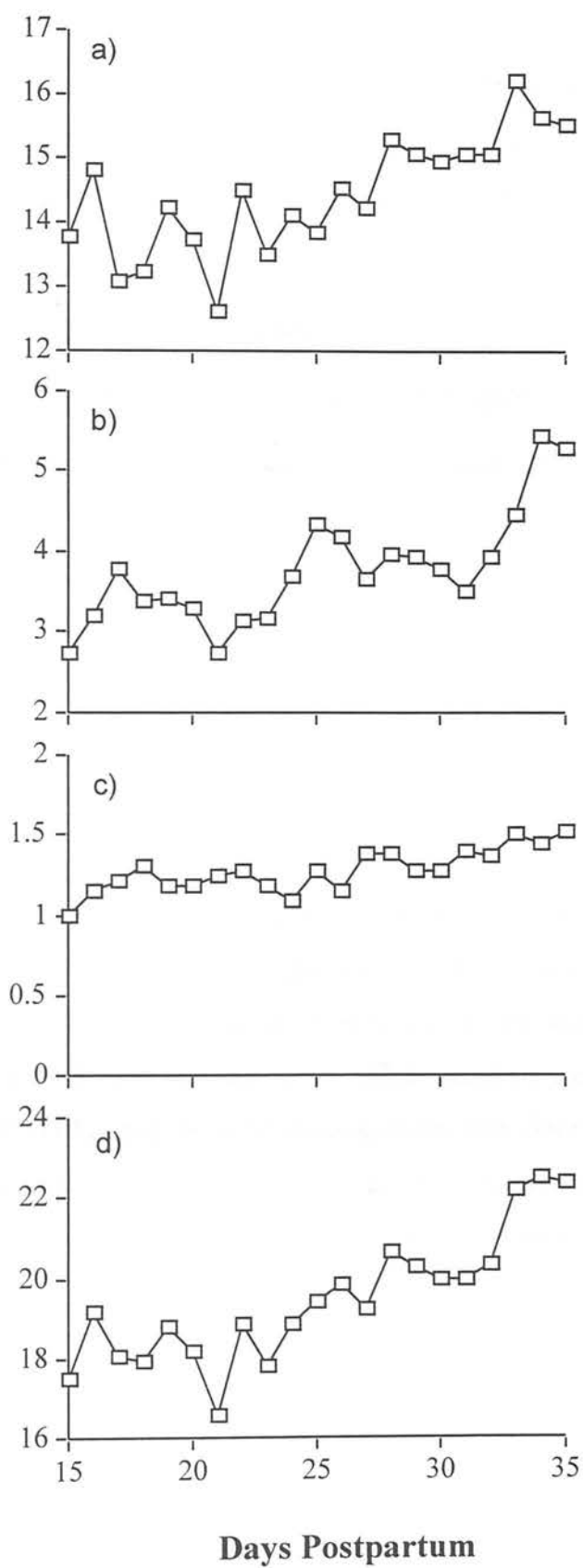


Table 5.3. Mean serum concentrations of GH, insulin, BOHB, urea and glucose between days 15 to 35 postpartum for cows from low or high genetic merit. a, b ($p<0.01$) and c, d ($p<0.06$) indicate significant differences within the same column.

	GH	insulin	BOHB	urea	glucose
Low	7.01±0.85 ^a	0.18±0.02 ^c	0.7±0.05 ^a	6.33±0.25 ^a	3.49±0.13 ^c
High	10.2±1.09 ^b	0.13±0.03 ^d	1±0.03 ^b	6.76±0.26 ^a	3.18±0.09 ^d

5.4.5 RELATIONSHIP BETWEEN METABOLIC HORMONES AND FOLLICULAR DEVELOPMENT

Correlation coefficients for the numbers of small, medium-sized and large follicles with PCBW, metabolic hormones (GH and insulin), and metabolites (BOHB, glucose and urea) are shown in Table 5.4. Predicted changes in body weight were highly correlated ($P<0.01$) with numbers of medium-sized follicles, but not with the number of small or large follicles ($P>0.10$). However, small follicle numbers were positively correlated with insulin ($P<0.05$), urea ($P<0.05$) and negatively with GH ($P<0.10$). For medium-sized follicle numbers, there was also a negative correlation with GH ($P<0.01$). Large follicles showed a positive correlation with glucose concentrations ($P<0.01$).

Figure 5.2. Mean numbers of small (a), medium-sized (b), large (c) follicles and FSH concentrations (d) on the days immediately before or after ovulation in the postpartum period between days 15 to 35 postpartum. SEM values were 1.79, 1.77, 0.38 and 0.14 for small, medium-sized, large follicle number and FSH concentrations respectively.

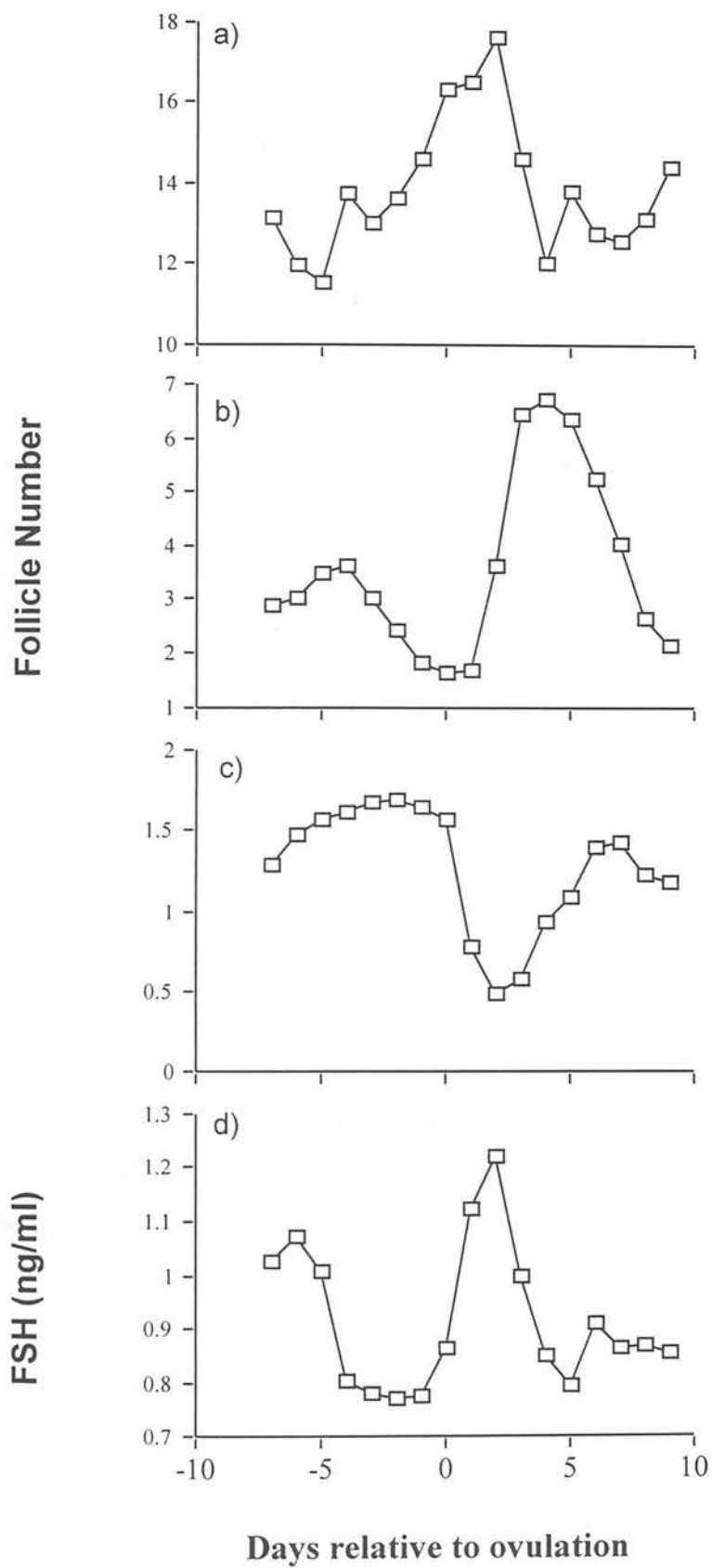


Table 5.4. Pearson correlation coefficients and corresponding (P) values for small, medium-sized and large follicle numbers with predicted changes in body weight (PCBW), GH, insulin, BOHB, urea and glucose.

	PCBW	GH	insulin	BOHB	urea	glucose
Small follicles	0.032	-0.069	0.079	0.015	0.108	0.017
P	0.392	0.067	0.035	0.776	0.040	0.740
Medium follicles	0.148	-0.132	-0.028	0.075	0.046	0.054
P	0.000	0.001	0.456	0.152	0.386	0.307
Large follicles	0.037	0.008	-0.045	-0.053	0.029	0.143
P	0.329	0.824	0.235	0.317	0.576	0.006

5.5 DISCUSSION

This study examined the effects of genetic selection for predicted breeding value for milk production on follicular dynamics during the early postpartum period. Cows selected for high milk production had a longer interval from parturition to first ovulation (27.2 ± 1.9 days) compared to cows selected for low milk yield (20.2 ± 1.6 days). Moreover, a higher proportion of L than H cows ovulated within the 35 days postpartum study period. Thus, the difference to first ovulation postpartum between lines is greater than that estimated here. This effect was independent of milk production, since it did not vary between lines during the study period.

Regardless of the genetic line, all cows already had active ovaries with all three size categories of follicles being present when the study started at day 15 postpartum. Follicular waves were observed during the study. Well fed dairy cattle normally contain active ovaries in the early postpartum period (Savio *et al.*, 1990; Beam and

Butler, 1997). However, under severe undernutrition, ovarian follicle selection can cease (Lucy *et al.*, 1991b; Rhodes *et al.*, 1995). Previous studies of folliculogenesis prior to ovulation reported that the numbers of small follicles decreased with advancing day postpartum and as ovulation approached (Lucy *et al.*, 1991a; Gutierrez *et al.*, 1994). These observations were consistent with the development of dominant preovulatory follicles. In the present study, small follicle numbers increased with day postpartum. This increase was not significant when predicted changes in body weight were included as a covariate in the analysis. This result indicates that changes in body weight (and therefore energy balance) strongly influences the size of the small follicle pool developing in the ovary and supports data showing an increment in small follicle numbers seen in overfed animals (Maurasse *et al.*, 1985; see Chapter 4).

Similarly, numbers of medium-sized follicles increased as lactation advanced, and their numbers correlated with predicted changes in body weight. Diets that improved energy balance were associated with increased numbers of medium-sized (5-9 mm) follicles (Lucy *et al.*, 1991a,b). It appears contradictory that treatment of dairy cattle with bovine somatotrophin (BST), which temporarily reduces energy balance whilst increasing milk production, also increased the numbers of small (<5mm) and medium-sized follicles (De la Sota *et al.*, 1993; Lucy *et al.*, 1993b; Herrier *et al.*, 1994; Kirby *et al.*, 1997). However, the effect of BST treatment on follicular development may be due to nutrient redistribution, resulting in higher blood glucose concentration (Bauman, 1992). In support of the theory that glucose redistribution may enhance follicular development, Downing and Scaramuzzi (1995) observed an increase in ovulation rate as a result of glucose infusion in sheep.

As expected, the presence of a dominant follicle (Table 5.2) caused a decrease ($p < 0.09$) in small follicle numbers. However, this only happened when PCBW was included as a covariate in the analysis. Thus, changes in body weight (or in factors correlated with body weight: *e.g.* days postpartum, insulin, glucose) affected the number of small follicles by a mechanism independent of follicular dominance. Nonetheless, small follicle numbers fluctuated in a wave-like fashion preceding the

wave of medium-sized follicles and immunoreactive FSH, even in the presence of a dominant follicle. The increase in small follicle numbers in the presence of the dominant follicle has not been detected previously by ultrasonography. However, previous studies have found that the number of small follicles reaches a peak on days 1 and 2 of the oestrous cycle (Matton *et al.*, 1981; Adams *et al.*, 1993; De la Sota *et al.*, 1993; Kirby *et al.*, 1997; see Chapter 4). Therefore, the wave of small follicle growth must begin in the presence of the dominant follicle. Nonetheless, despite the increase in small follicle number, the largest follicle appeared to remain dominant as medium-sized follicle numbers did not increase until after ovulation. Perhaps, small follicles can respond to a rise in FSH concentrations which is not detectable by RIA, or the increase in small follicle number may be stimulated by factors other than FSH in the presence of the dominant follicle. Alternatively, the greater number of small follicles may have corresponded to the number of atretic medium-sized follicles that decreased in size after failing to be selected. Indeed, Rajakoski (1960) reported an increase in the number of atretic follicles ($>1\text{mm}$) on the days immediately after the number of follicles $>5\text{mm}$ had reached a peak.

As expected, the presence of the dominant follicle during the postpartum period (Table 5.2), or in the preovulatory period (Figure 5.2), provoked a decrease in medium-sized follicle numbers and FSH concentrations. (Adams *et al.*, 1993; Fortune, 1994). The pattern of growth of medium-sized follicles agrees with previous reports (Adams *et al.*, 1994; Gong *et al.*, 1993a; De la Sota *et al.*, 1993; Fortune, 1994; Kirby *et al.*, 1997; Chapter 4) showing that numbers increased after the postovulatory FSH surge until a large dominant follicle was selected.

After parturition, lactational energy requirements are greater than voluntary food intake (Veerkamp *et al.*, 1994; Senatore *et al.*, 1996). Therefore, lactating cows are in negative energy balance and will use body energy reserves to sustain milk production. Parallel to the increase in milk yield, voluntary food intake increased (Butler and Smith, 1989; Veerkamp *et al.*, 1994; Senatore *et al.*, 1996) and energy balance recovered after a nadir occurring within two weeks after parturition (Lucy *et al.*, 1991a; Veerkamp *et al.*, 1994; Senatore *et al.*, 1996; Beam and Butler, 1997).

The reduction in reproductive performance in the postpartum period is attributed to decreased energy balance in highly productive animals (for review see; Butler and Smith, 1989; Swanson, 1989; Robinson, 1990; Jolly *et al.*, 1995). Specifically, first ovulation postpartum in dairy cattle occurs some days after the nadir in energy balance is reached (Zurek *et al.*, 1995; Senatore *et al.*, 1996; Beam and Butler, 1997). The main factor causing delayed ovulation postpartum is low LH pulsatility (Butler and Smith, 1989; Canfield and Butler, 1990; Robinson, 1996). Moreover, altered LH pulsatility is linked to nutritional changes. Thus, energy balance seems to be a major factor influencing reproductive performance in the postpartum lactating cow. Since cattle were managed under commercial conditions and kept in pasture in this study, energy balance could not be calculated. We opted instead to use changes in body weight as an indicator of energy balance. No differences in PCBW between the H and L lines were observed. Furthermore, predicted changes in body weight in both lines changed from negative at the beginning of the study, to positive towards the end of the study, with no differences between lines. Imakawa *et al.* (1986b) and Roberson *et al.* (1991) found that the direction of body weight changes affected the pattern of LH secretion in heifers, and heifers gaining weight had a higher LH pulse frequency than heifers losing weight. The lack of difference in body weight change and milk production during the study period indicates that no major differences in energy balance occurred between H and L cows.

The higher plasma concentrations of GH (Pell and Bates, 1990) and BOHB (Perotti *et al.*, 1989) together with lower insulin and glucose (Butler and Smith, 1989) concentration in H cows, indicate that H cows were in lower energy balance than L cows. However, a cautionary note should be sounded for this interpretation. Firstly, animals selected for higher milk yields have higher GH concentrations compared to cows with lower genetic value (Barnes *et al.*, 1985; Kazmer *et al.*, 1986; Bauman, 1992), and this difference in GH concentration is independent of changes in energy balance (Kazmer *et al.*, 1986). Furthermore, studies carried out in prepubertal heifers from these same lines showed that GH pulse amplitude in response to GH-releasing factor was higher in animals of high genetic merit than in their low genetic merit counterparts (Lovendahl *et al.*, 1991; Woolliams *et al.*, 1993). In addition, insulin

concentrations from selected cows were greater in response to feeding (Xing *et al.*, 1993), and insulin and glucose clearance time was longer (Barnes *et al.*, 1985) than non-selected cattle. Furthermore, high predicted breeding value-calves responded to fasting with higher free fatty-acid serum concentrations than those of low PBV (Woolliams *et al.*, 1992). Interestingly, insulin and glucose clearance time from circulation in feed-restricted heifers (Richards *et al.*, 1989b) and lambs (Cole *et al.*, 1993) was increased compared to well-fed heifers. Thus, the metabolism of selected cattle differs from that of non-selected cattle in a way that seems to make the animal better adapted to mobilise body energy reserves to deal with higher energy demands, such as lactation. These changes in metabolism, which ensure adequate nutrient availability for maintenance of milk production, may result on detrimental effects in reproductive function (Amaral-Phillips *et al.*, 1993; Swanson, 1989).

No data are available which investigate the interactions between metabolic adjustments occurring with selection and the ways in which the brain might interpret them. How the brain reads the metabolic status is unknown, although metabolic hormones and metabolites are supposed to play a primary role. Decreased insulin and/or glucose in sheep (Downing *et al.*, 1995; Downing and Scaramuzzi, 1997; Bucholtz *et al.*, 1996) and cattle (McClure, 1968; McClure *et al.*, 1978; McCann and Hansel, 1986) inhibited oestrous behaviour, ovulation, and caused a reduction in LH pulse frequency. Likewise, direct effects of metabolic hormones or nutrients at the follicular level cannot be excluded, as a rise in insulin was associated with increased numbers of small follicles *in vivo* (Gong *et al.*, 1993a; see Chapter 4). *In vitro*, insulin enhances theca cell (Spicer *et al.*, 1995b; Campbell *et al.*, 1996a) steroidogenesis and the stimulatory effect of FSH on granulosa cells (see Chapter 6, Campbell *et al.*, 1995). Glucose increased the ovulation rate in sheep (Downing *et al.*, 1995) and in this study glucose levels were correlated with the number of large follicles. *In vitro*, glucose stimulated bovine theca cell steroidogenesis (Spicer *et al.*, 1995b).

In agreement with the results of Chapter 4, GH was negatively correlated with increased numbers of small follicles. Since GH receptors have not been found in

follicular tissue (Lucy *et al.*, 1993b) and unluteinised granulosa cells are unresponsive to GH *in vitro* (unpublished observations), it would seem that GH is not directly implicated in follicular development.

In summary, selection on predicted breeding value for milk production was associated with a longer interval from parturition to first ovulation. This was independent of major changes in follicular development, milk production or changes in body weight. However, there were differences in blood concentrations of metabolic hormones and metabolites that were associated with selection, and which may directly or indirectly alter follicular function resulting in delayed ovulation on animals selected for higher milk production.

The results of this Chapter, together with those of Chapter 4, indicate that GH does not have a direct role in follicular development. The high insulin and glucose levels, observed in cows with low PBV were associated with a shorter interval from partum to first ovulation. However, although the last two Chapters (4 and 5) have highlighted possible important associations between metabolic hormones and follicular function *in vivo*, the specific roles of metabolic hormones on follicular function are difficult to assess due to the complex interactions between them. Cell culture can provide the avenue whereby putative factors involved in the control of follicular function can be studied. Therefore, the development of a culture system where cells maintain physiological characteristics similar to those observed *in vivo* will undoubtedly advance our understanding further.

6. DEVELOPMENT OF A LONG TERM BOVINE GRANULOSA CELL CULTURE SYSTEM

6.1 ABSTRACT

The objectives of this study were to develop a serum-free bovine granulosa cell culture system in which FSH-responsive oestradiol production could be induced and maintained, then to use this system to evaluate the effects of FSH, insulin, and IGF-I on steroidogenesis and proliferation of bovine granulosa cells from different sized follicle categories. In the presence of FSH, granulosa cells from small follicles differentiated *in vitro* and oestradiol secretion increased with time ($p < 0.01$) so that by the end of the culture period it was similar to that of cells from large follicles. Granulosa cells from medium-sized and large follicles secreted oestradiol throughout the culture period. Cells cultured in plasma-coated culture wells had an increased proliferative response, but lower oestradiol production compared to cells cultured under serum-free conditions ($p < 0.01$). Insulin promoted proliferation and oestradiol production by granulosa cells from the three follicle size categories ($p < 0.01$). Physiological concentrations of FSH induced proliferation and oestradiol secretion ($p < 0.01$) by granulosa cells in a dose-responsive manner. The inclusion of IGF-I in the culture system enhanced proliferation and oestradiol production ($p < 0.01$), even in the absence of gonadotrophic support, demonstrating the gonadotrophic characteristics of this growth factor.

In conclusion, these results demonstrate the development of a physiologically relevant culture system for bovine granulosa cells which permits the study of key factors controlling the differentiation and proliferation of bovine granulosa cells.

6.2 INTRODUCTION

The later stages of follicular growth and development are primarily regulated by gonadotrophins. Although early follicular development can progress independently

of gonadotrophins follicles are highly dependent on gonadotrophins to achieve ovulatory size successfully (McNeilly *et al.*, 1991). It has been established in cattle that FSH is secreted throughout the oestrous cycle in a wave-like pattern, with a peak of FSH closely preceding the recruitment of follicles (Gong *et al.*, 1991; Adams *et al.*, 1994; see Chapter 4 and 5). In heifers rendered hypogonadotrophic by infusion of GnRH agonist, follicle growth continues to around 8 mm in diameter in the absence of pulsatile LH secretion, but stops at 4 mm when FSH concentrations are also suppressed (Gong *et al.*, 1995; 1996a). The gonadotrophin dependence of follicles correlates closely with changes in the pattern of expression of P450-aromatase mRNA (P450a), P450-side-chain cleavage mRNA (P450scc), and LH receptor mRNA (Xu *et al.*, 1995a,b) in the granulosa cells of growing bovine follicles.

From the results in Chapters 4 and 5 it is evident that factors other than gonadotrophins play key roles in follicular development and that these effects may be independent of gonadotrophins (Adashi and Rohan, 1992). For example, insulin and IGF-1 are associated with enhanced follicular development *in vivo*. Pharmacological treatment of heifers with bovine somatotrophin provokes an increase in the number of small follicles, associated with an increase in circulating insulin (Gong *et al.*, 1993a; De La Sota *et al.*, 1993) and IGF-I concentrations (Gong *et al.*, 1993a). Under physiological conditions, increased food intake also enhances the number of small follicles, even though there is no alteration in circulating FSH concentrations. However, this effect is associated with physiologically significant increases in peripheral insulin concentrations (see Chapter 4).

The relationship between these endocrine and ovarian changes *in vivo* is complex (see Chapter 4 and 5). Cell culture provides an approach where the actions and interactions of specific factors can be determined. Previous efforts to develop a bovine granulosa cell culture system (Gong *et al.*, 1994; Saumande, 1991; Spicer *et al.*, 1993a; Vernon and Spicer, 1994) have been confounded by a consistent decline in oestradiol production with time in culture (Gong *et al.*, 1994; Saumande, 1991; Spicer *et al.*, 1993a; Vernon and Spicer, 1994; Luck *et al.*, 1990), with a progressive

increase in progesterone synthesis (Luck *et al.*, 1990), suggesting the onset of luteinisation.

As well as being able to maintain oestradiol production *in vitro*, granulosa cells should also remain responsive to FSH. In most previously-described culture systems bovine granulosa cells only respond to pharmacological doses of FSH (Gong *et al.*, 1994; Vernon and Spicer, 1994; Alpizar and Spicer, 1993; Langhout *et al.*, 1991). As far as we are aware, only one previous study has reported a culture system where bovine granulosa cells could be stimulated with low doses of FSH: however, even in this system the decline in oestradiol production could not be prevented (Berndtson *et al.*, 1995).

Studies of growth factor effects require the use of a completely defined culture medium which does not contain serum. Previously, bovine granulosa cell culture systems have either added serum to the medium during the first hours of culture, or coated the plates with serum or other adhesion factors (Gong *et al.*, 1994; Spicer *et al.*, 1993a; Luck *et al.*, 1990; Langhout *et al.*, 1991; Wrathall and Knight, 1993; Roberts and Echterkamp, 1994) to facilitate the adhesion of the cells to the plate surface. As well as adding known serum factors, this approach also opens up the possibility of the introduction of unknown factors into the culture which may affect cellular responsiveness to subsequent stimuli.

In this chapter, a serum-free culture system for bovine granulosa cells is described in which oestradiol production can be both induced and maintained in response to physiological concentrations of FSH. This culture system has then been used to evaluate and compare the effects of FSH, insulin, and IGF-I on steroidogenic capacity and proliferation of bovine granulosa cells from follicles of different stages of development (small <4mm, medium-sized 4-8mm and large >8mm diameter).

6.3 MATERIALS AND METHODS

6.3.1 GRANULOSA CELL CULTURE

Granulosa cells were cultured in 96 well plates as described in Chapter 2 in the complete absence of serum, except where indicated. Between 50 to 75 X 10³ viable cells were seeded into each well and cultured for up to 6 days, replacing 70% of the spent media with fresh media every 48 h. The effects of serum-coating the wells, and of different concentrations of insulin (0-5000 ng/ml), FSH (0-100 ng/ml), IGF-1 (0-100 ng/ml) and Long-R3 IGF-1 (0-10 ng/ml) on granulosa cell proliferation and steroid production were tested. The inter- and intra-assay coefficients of variation were 9.6 % and 7.4 % for oestradiol and 4.8 % for progesterone assays.

6.3.2 STATISTICAL ANALYSES

Data are presented as least squares means and standard error of the mean (SEM) of four replicates per treatment from each of at least three separate cell culture experiments. Effects of time and treatment (insulin, IGF-I, FSH, and serum coating) were analysed using mixed models, allowing for variation due to culture and different pools of cells (random effects). To correct for heterogeneity of variance, oestradiol and progesterone production were analyzed after logarithmic transformation of hormone produced (natural log(hormone+1)) per day by 10⁴ cells. Cell number at the end of culture was analysed with the number of cells seeded as a covariate in the analysis. The plating density was 62±4.7, 63.8±4.7 and 75.9±4.7 X 10³ cells from small, medium-sized and large follicles respectively. The relationship between oestradiol and progesterone production in time in culture was analysed by regression analysis.

6.4 RESULTS

6.4.1 TIME COURSE

Oestradiol. After 48 h of culture, granulosa cells from follicles of all sizes, secreted substantial amounts of oestradiol. Cells from large follicles secreted five times (295 ± 6 pg/day/ 10^4 cells) higher quantities of oestradiol ($p < 0.01$) compared to cells from medium-sized follicles (44.7 ± 6 pg/day/ 10^4 cells). These in turn secreted twice the amount of oestradiol than granulosa cells from small (24.04 ± 6 pg/day/ 10^4 cells) follicles ($p < 0.05$). Overall, oestradiol production was maintained throughout the 6 days of culture. However, the secretion of oestradiol varied with duration of culture according to the origin of the cells and the treatment applied ($p < 0.01$). In the presence of 10 ng ml^{-1} insulin alone, oestradiol production by cells from small and medium-sized follicles was maintained throughout the culture period (time per size $p > 0.05$), whereas oestradiol production by cells from large follicles declined between 48 to 96 h of culture, but then increased again during the last period of culture ($p < 0.01$, Figure 6.1). The addition of FSH to the cells from small follicles stimulated a three-fold increase in oestradiol production with time ($p < 0.01$), but had little effect on oestradiol production by cells from medium-sized and large follicles ($p < 0.01$). In contrast, LR3-IGF-I in the absence of FSH stimulated oestradiol production by granulosa cells from small follicles 6-fold after 144 h in culture ($p < 0.01$), and maintained oestradiol production by granulosa cells from medium-sized follicles, but could not prevent the decline in oestradiol production by cells from large follicles ($p < 0.01$). In the presence of LR3-IGF-I, FSH had no effect on oestradiol production by granulosa cells from small and medium-sized follicles, but attenuated the decline in oestradiol production from 48-96 h of culture by cells from large follicles (Figure 6.1).

Progesterone. In a similar manner to oestradiol, progesterone production after 48 h of culture in the presence of insulin, LR3-IGF-I and FSH was highest in cells from large follicles and lowest in cells from small follicles (Table 6.1, $p < 0.01$). After 96 h, progesterone production by granulosa cells from all three follicle size categories had

increased two- to three-fold ($p<0.01$), and this rate of secretion was maintained throughout the rest of the culture.

In granulosa cells from small and medium-sized follicles there was a positive relationship between oestradiol and progesterone production with time (regression coefficients=0.78, $p<0.05$; and 1.8, $p<0.01$ for progesterone and time respectively). However, the change in the ratio of oestradiol:progesterone production by cells from small follicles (Table 6.1) indicated a larger increase in oestradiol production by these cells with time in culture than with cells from medium-sized follicles. In contrast, cells from large follicles showed an inverse relationship between oestradiol and progesterone production (regression coefficient= -1.15, $p<0.05$), with the ratio of oestradiol:progesterone significantly diminishing ($p<0.01$) with time in culture (Table 6.1).

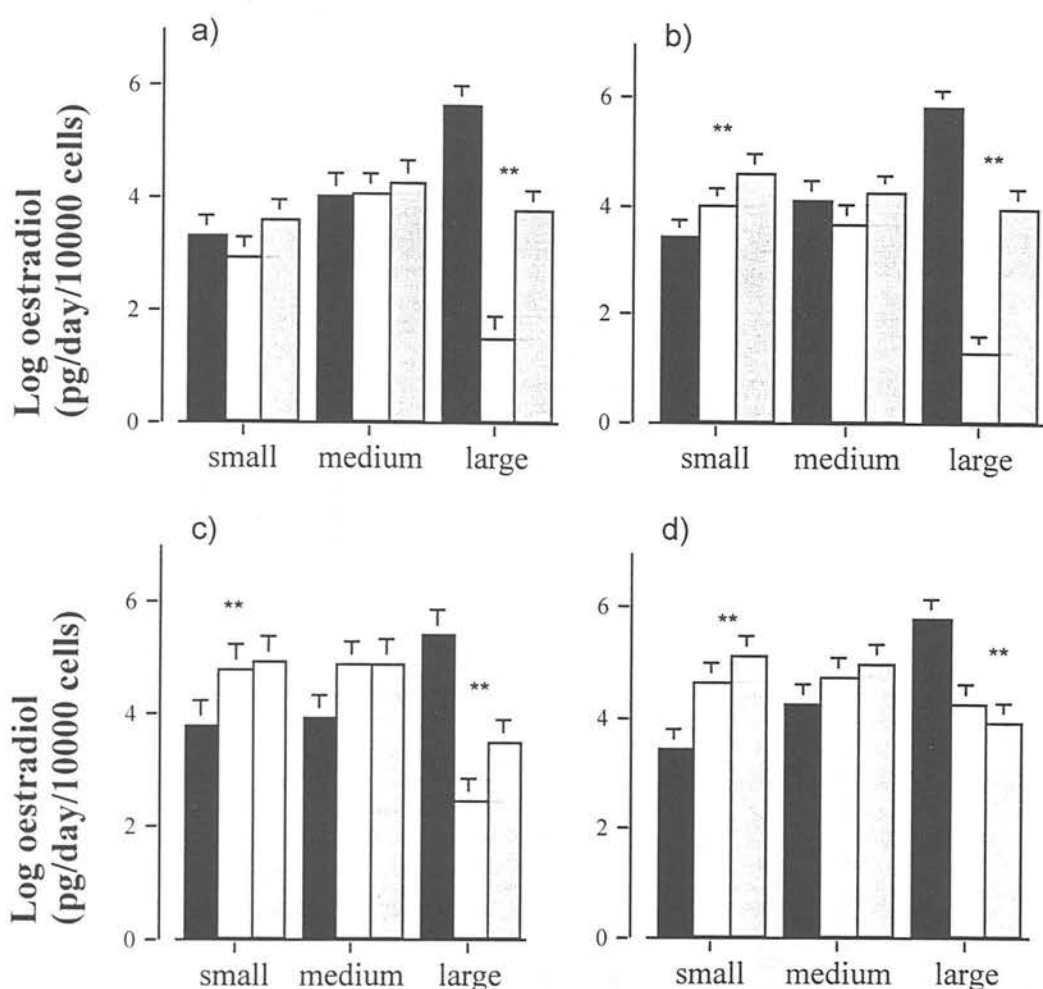


Figure 6.1. Oestradiol production per 10,000 bovine granulosa cells from small (<4 mm diameter), medium-sized (4-8 mm) and large (>8 mm) follicles after 0-48 (■), 48-96 (□) and 96-144 h (▨) of culture with either (a) 10 ng ml⁻¹ of insulin, (b) 10 ng ml⁻¹ of insulin and 1 ng ml⁻¹ bFSH-I-2, (c) 10 ng ml⁻¹ insulin and 10 ng ml⁻¹ LR3 IGF-1, or (d) insulin 10 ng ml⁻¹, LR3-IGF-I 10 ng ml⁻¹ and FSH 1 ng ml⁻¹. Values are least square means \pm SEM. The asterisk depicts significant differences (* p<0.01) with the 0-48h period within the same follicle-size category.

Table 6.1. Progesterone (progesterone log pg/day/10,000 cells), and ratio of oestradiol: progesterone production (e2/p4) by bovine granulosa cells from small (<4 mm), medium-sized (4-8 mm) and large follicles (>8 mm), in the presence of 10 ng of LR3 IGF-1 ml⁻¹, 10 ng insulin ml⁻¹ and 1 ng bFSH-I-2 ml⁻¹ in serum free conditions, after 0-48, 48-96 and 96-144 h of culture.

time in culture	<u>Follicle Size</u>			progesterone e2/p4	progesterone e2/p4	progesterone e2/p4	progesterone e2/p4	SEM
	<u>small</u>	<u>medium</u>	<u>large</u>					
0-48 h	6.96 ^a	0.49 ^a	7.65 ^a	0.54 ^a	7.93 ^a	0.76 ^a	0.3	0.05
48-96 h	8.00 ^b	0.54 ^{ab}	8.36 ^b	0.52 ^a	9.10 ^b	0.47 ^b		
96-144 h	7.85 ^b	0.61 ^b	8.68 ^b	0.55 ^a	9.15 ^b	0.45 ^b		

The SEM is 0.3 for progesterone and 0.05 for e2/p4 ratio.

Different letters within the same column differ at p<0.01.

6.4.2 EFFECTS OF SERUM

Seeding cells into serum-coated wells enhanced the number of cells at the end of culture ($p < 0.01$) in all three follicular size categories (Figure 6.2). However, oestradiol production between 96-144 h in culture was significantly reduced in cells from small ($p < 0.01$) and medium-sized follicles ($p < 0.05$), compared with cells cultured under serum-free conditions (Figure 6.2). In contrast, there was no effect of serum on progesterone production ($p > 0.05$) by cells from all three follicle size categories (Figure 6.2).

6.4.3 EFFECTS OF IGF-I AND IGF-I ANALOGUE (LR3-IGF-I)

Bovine granulosa cells from small and medium-sized follicles exhibited an increased proliferative response to LR3-IGF-I (1 ng ml^{-1} ; $P < 0.01$); whereas cells from large follicles required a dose of 10 ng ml^{-1} . Cells from all three follicle size categories showed a significantly increased proliferative response to IGF-I, but only at the highest dose (100 ng ml^{-1} ; Figure 6.3). Similarly, oestradiol production by cells from all size categories was enhanced by addition of IGF-I to the culture. Furthermore, in all cases the cells appeared to be more sensitive to IGF-I in terms of oestradiol production compared to cell proliferation, with significant ($p < 0.01$) increments in oestradiol secretion at doses as low as 0.1 ng ml^{-1} of LR3-IGF-I. In contrast, doses ten times greater were required for significant stimulation of cell proliferation. The LR3-IGF-I analogue was approximately 10 times more potent than the native hormone in stimulating both proliferative and oestrogenic responses by cells from all three follicle size categories.

6.4.4 EFFECTS OF INSULIN ALONE AND INTERACTIONS WITH IGF-I

In the absence of insulin, cell number at the end of culture fell below the initial plating density. The addition of insulin stimulated cell proliferation (Figure 6.4) in a dose-dependent manner ($p < 0.01$). The response of cells from all three follicle size categories was similar, but granulosa cells from small follicles had a greater

proliferative capacity ($p < 0.05$) than cells from medium-sized follicles, which in turn showed a greater response ($p < 0.05$) than cells from large follicles.

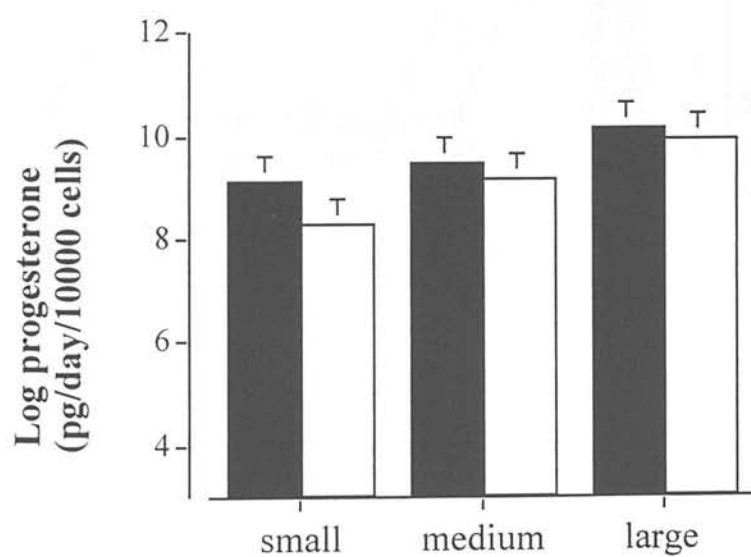
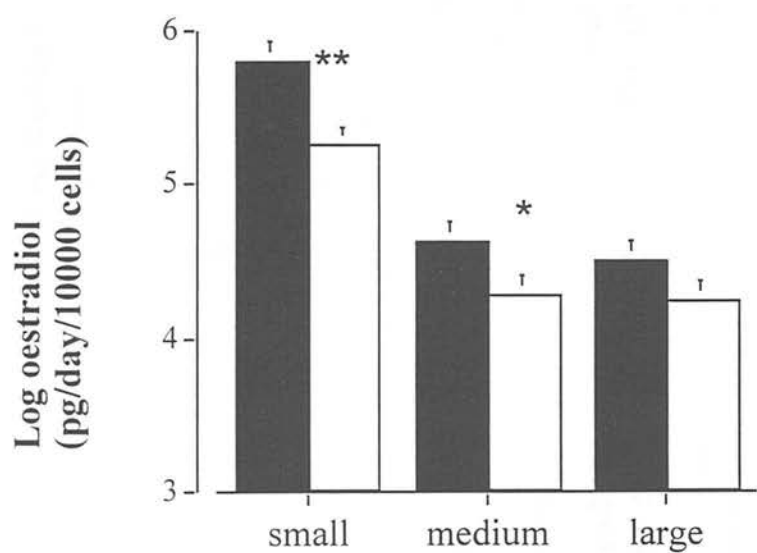
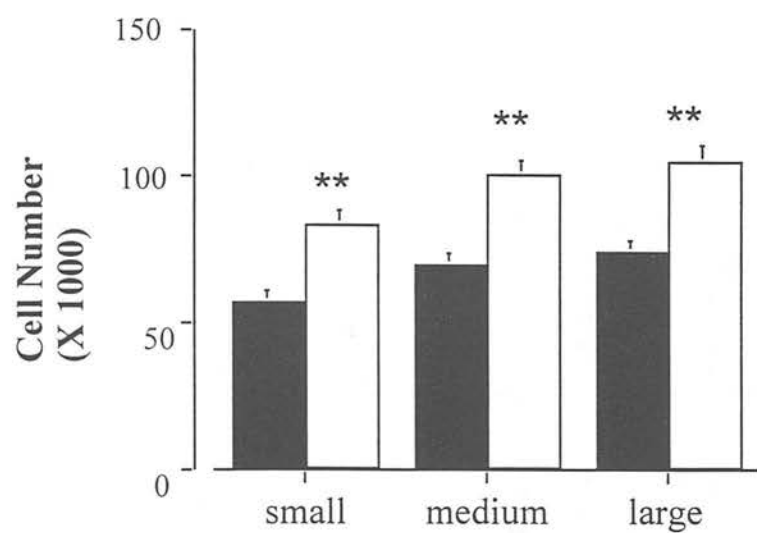
Insulin also stimulated oestradiol secretion in a dose responsive manner, oestradiol secretion increasing with increasing dose of insulin, and reaching a plateau at an insulin concentration of 100 ng ml^{-1} ($p < 0.01$). The addition of LR3-IGF-I, at doses of 1 ng ml^{-1} or greater, stimulated both cell proliferation and oestradiol production ($p < 0.01$), even in the absence of insulin. In the presence of IGF-I, the addition of insulin did not stimulate either cell proliferation or oestradiol production further ($p > 0.05$).

6.4.5 EFFECTS OF FSH

The effects of FSH on cell proliferation and oestradiol production are shown in Figure 6.5. FSH stimulated an increase ($p < 0.01$) in cell number when cultured in the presence of insulin (10 ng ml^{-1}) for 6 days, regardless of the size of the follicle from which the cells originated. When cells were cultured with 10 ng ml^{-1} of LR3-IGF-I alone, cell number also increased, and could not be increased further by addition of FSH. The combination of insulin (10 ng ml^{-1}) and IGF-I (10 ng ml^{-1}) had similar effects to IGF-I alone, although in granulosa cells from large follicles the combined treatment accentuated the stimulatory effect of FSH ($p < 0.05$) on cell proliferation.

FSH stimulated oestradiol production by cells from all three follicle size categories in a dose-dependent manner ($p < 0.01$) when cultured in the presence of 10 ng ml^{-1} of insulin (Figure 6.5). The response was linear up to a dose of 1 ng ml^{-1} , with oestradiol production being maintained by FSH concentrations up to 10 ng ml^{-1} . However, higher concentrations of FSH (up to 100 ng ml^{-1}), significantly ($p < 0.01$) decreased oestradiol production (data not shown). The inclusion of IGF-I, alone or in combination with insulin, stimulated maximal oestradiol production, even in the absence of FSH. At FSH doses higher than 1 ng ml^{-1} there was a decline in oestradiol production by granulosa cells from medium-sized and large follicles ($p < 0.05$).

Figure 6.2. Cell number at the end of culture and oestradiol and progesterone production per 10,000 bovine granulosa cells from small, medium-sized and large follicles cultured for up to 144 h, in the presence of 10 ng of LR3-IGF-1 ml⁻¹, 10 ng insulin ml⁻¹ and 1 ng bFSH-I-2 ml⁻¹. Cells were cultured either in serum-free conditions (■), or in serum-coated wells (□). Values are least square means \pm SEM. Asterisks depict significant differences (* $p < 0.05$, ** $p < 0.01$) within the same follicle-size category.



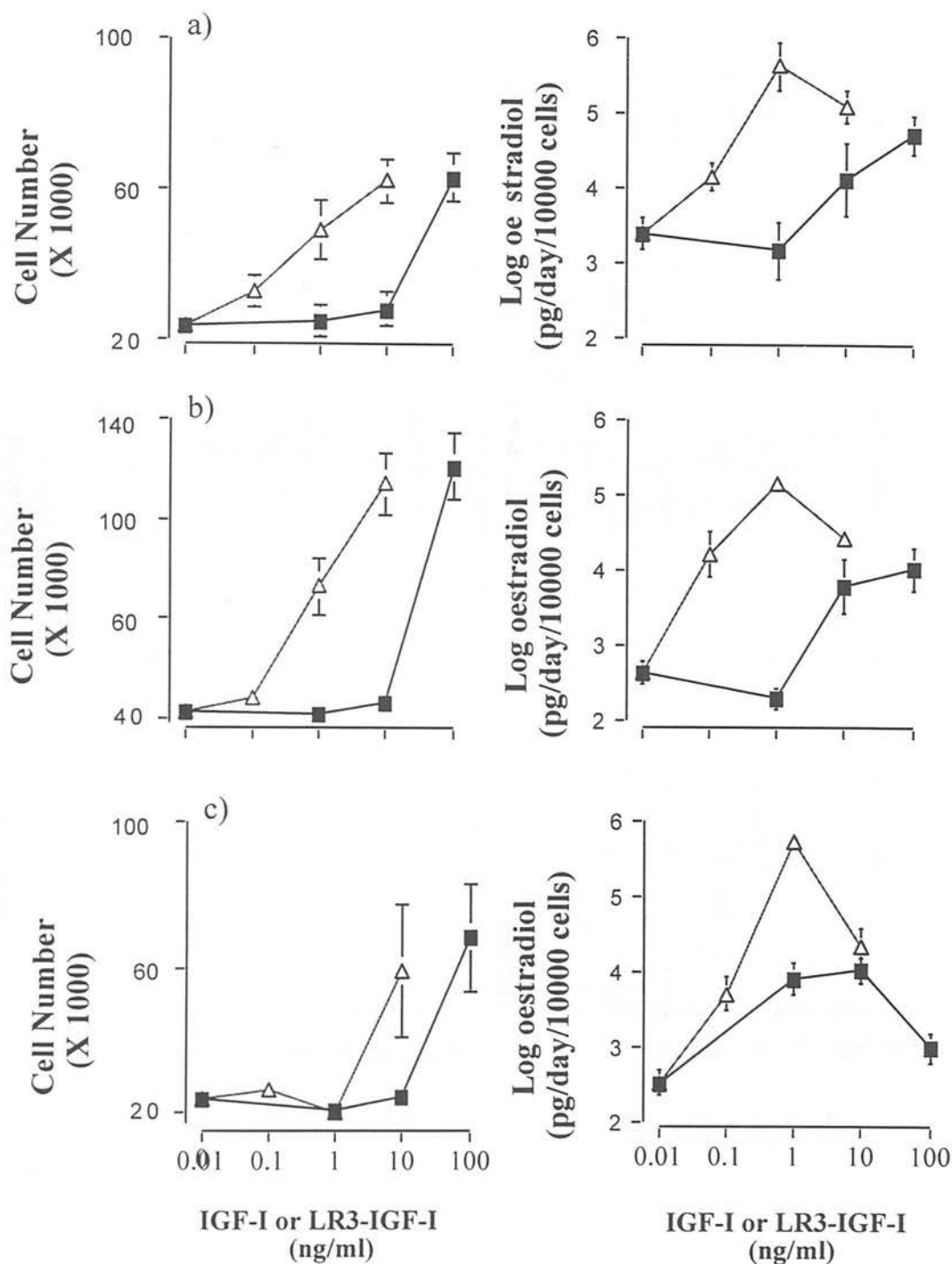


Figure 6.3. Cell number and oestradiol production per 10,000 bovine granulosa cells from (a) small, (b) medium-sized and (c) large follicles cultured for up to 144 h with either the IGF-I analogue LR3-IGF-I(Δ), or IGF-I (■) in the presence of 1 ng ml^{-1} of FSH. Values are least square means \pm SEM.

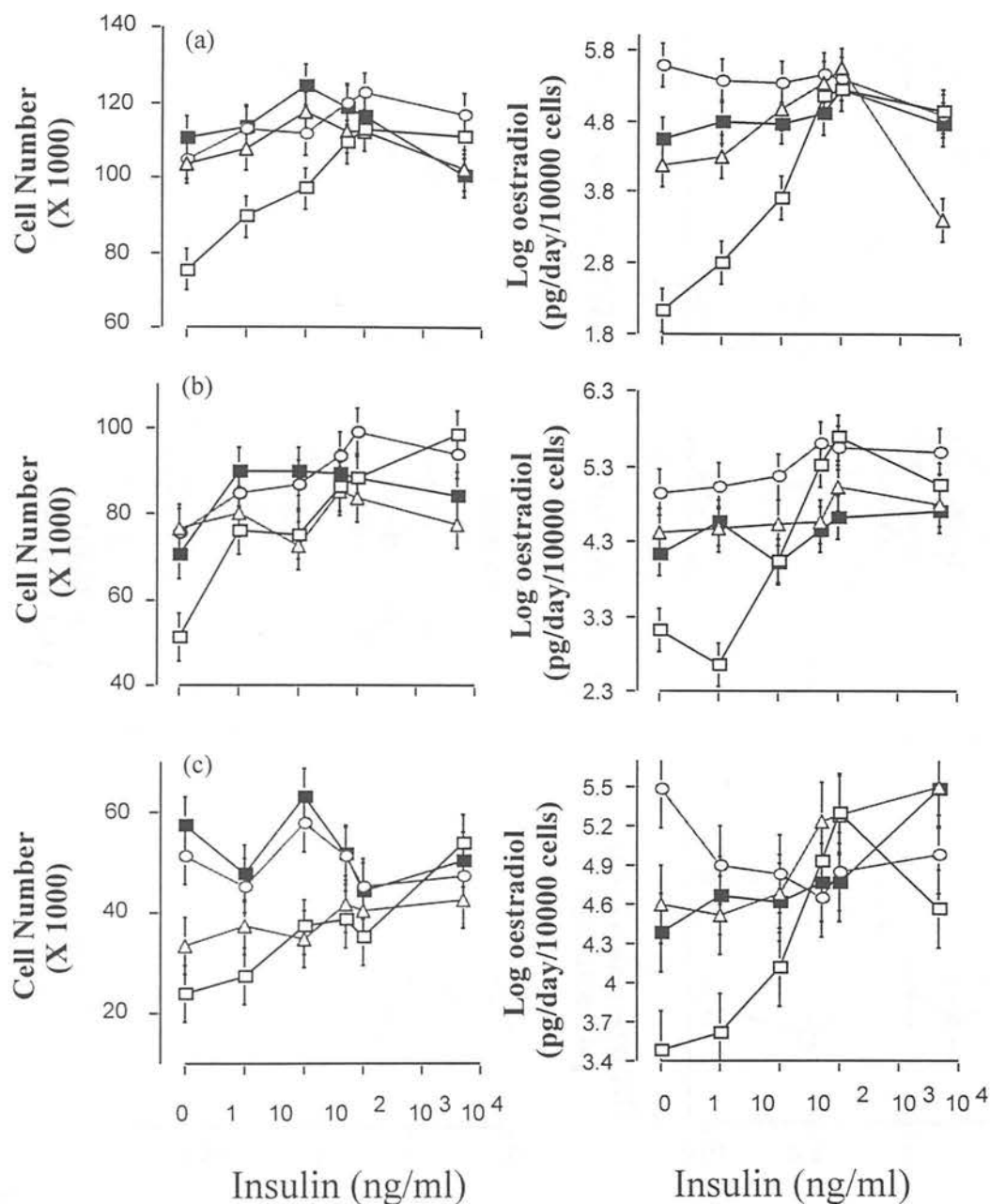


Figure 6.4. The effects of insulin on cell proliferation and oestradiol production by bovine granulosa cells of (a) small, (b) medium-sized and (c) large follicles after 144 h in serum free culture, in the presence of 0 (\square), 1 (\triangle), 10 (\circ) and 100 (\blacksquare) ng ml⁻¹ of LR3-IGF-I. All cells received 1 ng ml⁻¹ of FSH.

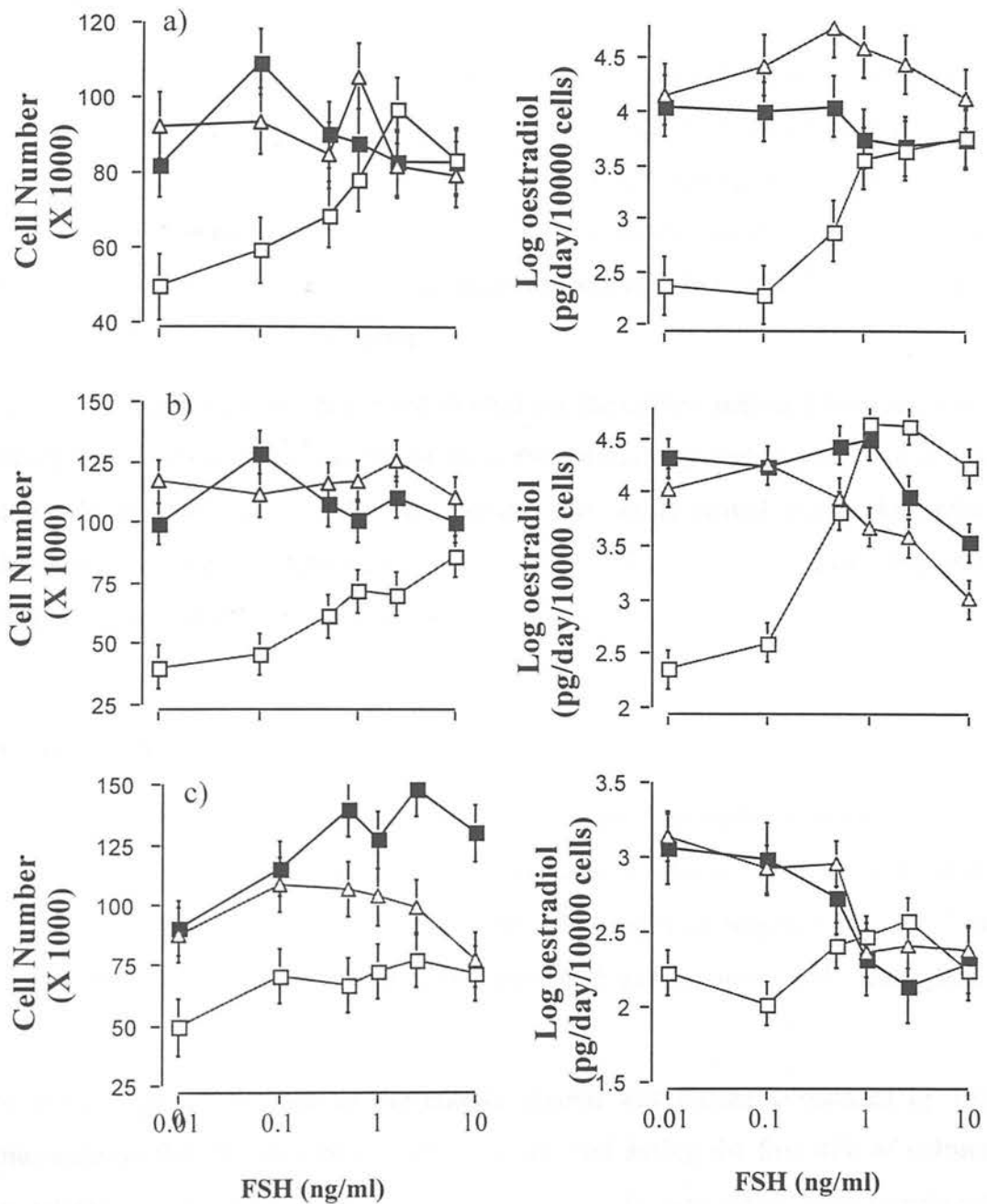


Figure 6.5. The effects of FSH on cell proliferation and oestradiol production by bovine granulosa cells from small, medium-sized and large follicles cultured for up to 144 h in serum free culture medium. The medium contained either 10 ng ml⁻¹ insulin (□), 10 ng ml⁻¹ LR3-IGF-I (Δ), or a combination of insulin and LR3-IGF-I (■). Values are least square means \pm SEM.

6.4.6 CELL MORPHOLOGY

Granulosa cells from all three size categories were seeded as an homogenous suspension of cells that initially covered the entire surface of each culture well. After 48h in culture, cells were grouped in tightly-formed aggregates, in which the spherical appearance of granulosa cells *in vivo* was conserved (Figure 6.6). These aggregates were attached to the culture plate by enlarged, flattened fibroblastic-like cells that formed the feet of the clump.

This basic morphology was preserved throughout the culture period. However, FSH ($>10 \text{ ng ml}^{-1}$) increased the number of these fibroblastic-like cells by the end of the culture. In addition, when cells were seeded into serum-coated wells, the entire culture well surface was covered by the fibroblastic-like cells, on top of which the clumps of spherical cells were attached.

6.5 DISCUSSION

The results of these studies demonstrate the successful development of a serum-free culture system for bovine granulosa cells in which oestradiol production can be both induced and/or maintained. Furthermore, the cells remained responsive to FSH at physiological concentrations, with both increased proliferative and oestrogenic responses.

The physiological relevance of the culture system was further confirmed by the demonstration that the amount of oestradiol secreted during the first 48h of culture was related to size of the follicle from which the cells originated, with increasing amounts of oestradiol production accompanying an increase in follicle size. Importantly, these results suggest that granulosa cells from small follicles can mature *in vitro* to a stage where they secrete similar quantities of oestradiol to more differentiated cells obtained from large follicles. This is supported by the result that after 48 h in culture, FSH increased oestradiol production by cells from undifferentiated follicles (small: $<4\text{mm}$) by 6-fold. Progesterone was also increased by 3-fold, but only after 144h in culture. Significant P450-aromatase and P450 side-

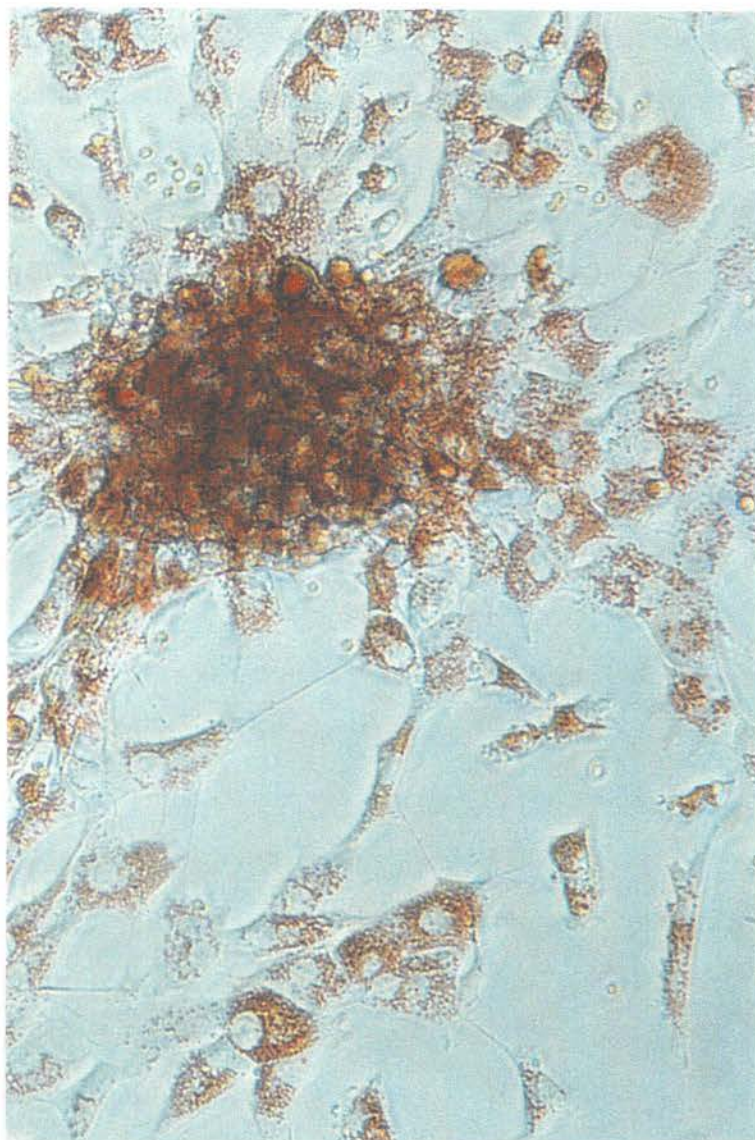


Figure 6.6. Morphological appearance of bovine granulosa cells after 144h in serum free culture, in the presence of 10 ng of LR3-IGF-1 ml⁻¹, 10 ng insulin ml⁻¹ and 1 ng bFSH-I-2 ml⁻¹. Cells in culture were characterized by the formation of tight aggregates (clumps) of spherical cells growing on top of enlarged, flattened fibroblastic-like cells. X 400.

chain cleavage expression has been shown to be absent from bovine granulosa cells from follicles < 4 mm in diameter (Xu *et al.*, 1995a). Cell differentiation *in vitro* has also been observed by ovine granulosa cells (Campbell *et al.*, 1996a), but the steroidogenic capacity of the sheep granulosa cells from small follicles (<3.5mm) after 6 days of culture was still not as great as cells from large (>3.5 mm) ovine follicles. In addition to their increased steroidogenic capacity, cells from small bovine follicles had a higher proliferative response to FSH, insulin and IGF-I compared with cells from large bovine follicles. This pattern is similar to that seen in mice, where granulosa cell proliferative capacity decreased with increasing steroidogenic activity (Hirshfield, 1991). The time course of granulosa cell differentiation in this bovine culture system appeared to parallel the situation *in vivo*. A period of about 4 days is required for cells to proliferate and differentiate *in vitro*, and this corresponds with the length of time taken by a recruited follicle to grow and differentiate into an ovulatory follicle in cattle (Gong *et al.*, 1991; Stock and Fortune, 1993; Turzillo and Fortune, 1993). The differential pattern of oestradiol secretion by bovine granulosa cells from different follicle size categories indicates that this culture system is an appropriate model for the study of differentiation of granulosa cells *in vitro*.

There was a decline in oestradiol production with time in culture by granulosa cells from large follicles (>8 mm). Although these cells still secreted considerable amounts of oestradiol at the end of culture, the ratio of oestradiol to progesterone secretion decreased with time, in contrast to cells from small and medium-sized follicles, where the ratio either did not change (medium-sized) or even increased (small). This change in the pattern of steroid production has been found in differentiated cells from sheep (Campbell *et al.*, 1996a) and cattle (Wrathall and Knight, 1993; Berndtson *et al.*, 1995), but it may not necessarily imply luteinisation, as the cells were still responsive to FSH.

Previous studies have failed to demonstrate stimulatory effects of FSH on the proliferation of bovine granulosa cells in culture (Langhout *et al.*, 1991; Gong *et al.*, 1993c). Contrary to these previous reports, FSH in the presence of 10 ng ml⁻¹ of

insulin stimulated proliferation of cells from all three follicle size categories. This observation agrees with findings of Campbell *et al.* (1996), where physiological doses of FSH stimulated ovine granulosa cell proliferation *in vitro*. Moreover, in the present study both cell proliferation and oestradiol production were enhanced by FSH. Cells were also more sensitive to FSH in terms of oestradiol production compared to cell proliferation (Figure 6.5), to a dose of 1 ng ml⁻¹ FSH stimulating maximal oestradiol production compared with a dose of 10 ng ml⁻¹ FSH for induction of maximal cell proliferation. In addition, the dose of 1 ng ml⁻¹ FSH required to obtain maximum stimulation of oestradiol production was 10 times lower than that required for sheep granulosa cells under similar conditions (Campbell *et al.*, 1996a).

IGF-I stimulated an increase in both cell number and oestradiol production, even in the absence of FSH, demonstrating the gonadotrophic action of this growth factor (Adashi and Rohan, 1992). Insulin had a dose-dependent stimulatory effect *in vitro* on granulosa cell numbers with cells from all three follicle size classes (Saumande, 1991; Langhout *et al.*, 1991; Peluso *et al.*, 1995). In addition, both insulin and IGF-I stimulated oestradiol secretion in a dose-dependent manner. While stimulatory effects of IGF-I on granulosa cell aromatase activity and cell proliferation have been described in several species (Adashi and Rohan, 1991; Campbell *et al.*, 1996a; Howard and Ford 1994), inhibitory effects of IGF-I on oestradiol production have also been reported (Spicer and Echterkamp, 1995). The inclusion of IGF-I in this culture system at doses as low as 1 ng ml⁻¹ stimulated oestradiol production and proliferation to levels equal to the highest levels reached with insulin alone. Addition of insulin had no further stimulatory effect on either oestradiol or proliferative responses. The stimulatory effects of IGF-I on both steroidogenesis and cell proliferation differ from those seen in ovine granulosa cells, where IGF-I had additive effects with both insulin and FSH (Campbell *et al.*, 1996a). The increased sensitivity to IGF-I of bovine granulosa cells compared with sheep, suggests that bovine follicles may have tighter regulatory control of the IGF-I system than in sheep.

The finding that low concentrations of IGF-I can stimulate oestradiol production and cell proliferation by bovine granulosa cells independently of gonadotrophins, highlights the potential action of this peptide as an intra-ovarian regulator of follicular function. Similar results were obtained with higher doses of insulin (50 ng ml⁻¹ or above). This may explain the failure of previous investigators to observe any positive response to FSH stimulation with cultures containing pharmacological amounts of insulin (Saumande, 1991; Langhout *et al.*, 1991; Berndtson *et al.*, 1995). It has been suggested that insulin at these high concentration acts through the IGF-I type I receptor (Kahn *et al.*, 1981; Poretsky and Kalin, 1987). This view is further supported by the comparable levels of stimulation of granulosa cell proliferation and steroidogenesis by either high concentrations of insulin or lower concentrations of IGF-I, and the failure to stimulate these cells further when insulin and IGF-I were added in combination (Figure 6.4).

The difference in sensitivity of granulosa cells to native IGF-I and IGF-I analogue highlights the importance of the IGF-binding proteins in regulating the local actions of IGF-I. The IGF-I analogue, LR3-IGF-I, was 10-fold more potent than the native form of the hormone in stimulating both cell proliferation and oestradiol production by granulosa cells. This is similar to a previous report for porcine granulosa cells (Howard and Ford, 1994). This higher potency *in vitro* is presumably due to the reduced affinity of LR3-IGF-I for IGF-I binding proteins (IGFBPs), which are produced in large quantities by granulosa cells cultured under these conditions (Armstrong *et al.*, 1996b,c).

The mechanisms through which IGF-I stimulates granulosa cell proliferation (Campbell *et al.*, 1996a; Giudice, 1992; Jones and Clemmons, 1995; Monget *et al.*, 1993) and differentiation (Adashi and Rohan, 1992; Giudice, 1992; Jones and Clemmons, 1995) are unknown. It has been hypothesized that IGF-I serves as an amplifier of gonadotrophin action in the ovary (Adashi and Rohan, 1992). However, whether IGF-I is synthesized by granulosa cells from ruminants is still not proven. A recent report in sheep, using *in situ* hybridisation, suggested that granulosa cells produce IGF-I (Leeuwenberg *et al.*, 1995). However, other researchers have been

unable to detect IGF-I mRNA expression using similar techniques (Perks *et al.*, 1995). In cattle, we have been unable to demonstrate the presence of IGF-I or its mRNA in granulosa cells either before or after culture (see Chapter 10), although IGF-I mRNA expression was found in bovine thecal cells. Furthermore, no temporal relationship has been found between IGF-I levels in the follicular fluid and the health or stage of development of follicles in cattle (Stock and Fortune, 1993; Leeuwenberg *et al.*, 1996). It is likely that extra-ovarian sources of IGF-I enhance follicular growth and steroidogenesis (Campbell *et al.*, 1995), and account for some of the pharmacological effects of BST (Gong *et al.*, 1993a), and some of the physiological effects of nutrition (Downing and Scaramuzzi, 1991; see Chapter 4) on follicular development.

Few reports are available on the role of IGF-I on ovarian function *in vivo*. Indirect evidence in favour of a role of IGF-I and/or insulin in follicular development *in vivo* comes from studies using BST. BST increases circulating IGF-I and insulin concentration in non-lactating cattle, and enhances follicular development (Gong *et al.*, 1993a,b). Manipulation of insulin, IGF-I and GH through changes in diet may also explain some of the effects of nutrition on ovarian function (see Chapter 4). Since the number of small follicles growing in the ovary was inversely correlated with GH concentration (see Chapter 4), it is likely that changes in follicular growth are due to the action of IGF-I and/or insulin. Whilst stimulatory effects of IGF-I on follicular growth and steroidogenesis have been demonstrated *in vivo* (Campbell *et al.*, 1995), exogenous insulin failed to induce a similar stimulation (Downing and Scaramuzzi, 1991; Simpson *et al.*, 1994). Therefore, more detailed investigation is needed to determine the precise roles of insulin and IGF-I on follicular development *in vivo*.

In the culture system reported here, oestradiol was secreted by cells cultured in serum-coated wells: however, oestradiol secretion corrected for cell number was significantly lower than for cells cultured under serum free-conditions. This is in contrast to the previous results of Berndtson *et al.* (1995) where addition of serum appeared to have a positive effect on the stimulation of oestradiol production,

although the amount of oestradiol that the granulosa cell produced was not corrected for cell number. In support of previous results (Langhout *et al.*, 1991), serum caused an increase in proliferation of the cells in culture (Figure 6.2). In contrast to oestradiol, progesterone was unaffected by serum pretreatment (Figure 6.2). This is of interest, as this result suggests that reduced oestradiol production by cells cultured with serum may not be due to luteinisation, but rather to an overall reduction in the steroidogenic capacity of the cells.

Granulosa cell organisation in culture was characterized by the formation of tight clumps (Figure 6.6), formed by cells with spherical morphology that resembles that of granulosa cells within the follicle. The intimate contact between cells observed in this culture system could be, at least in part, responsible for the successful maintenance of the aromatase activity and FSH responsiveness during culture. It has been suggested that cell to cell interaction becomes of primary importance during antrum formation of the follicle and that gap junctions attain their maximum size and frequency in the mature preovulatory follicle (Amsterdam and Rotmensch, 1987). Moreover, alterations in cell shape and size in response to FSH have been described previously (Amsterdam and Rotmensch, 1987). At the base of the clumps, enlarged, fibroblastic-like cells were observed. In serum-coated wells, and in response to high doses of FSH, there was an increase in the proportion of fibroblastic-like cells attached to the culture well surface. These observations, coupled with the observed overall decrease in steroidogenesis of granulosa cells cultured on serum coated-wells, suggests an important structure:function relationship.

Despite the many superficial similarities between ovine and bovine granulosa cells in culture, this study has highlighted some important species differences in the responsiveness of ovine (Campbell *et al.*, 1996a) and bovine granulosa cells. Firstly, bovine granulosa cells are more sensitive than ovine granulosa cells to both FSH and IGF-I stimulation at all stages of follicle development. Secondly, the steroidogenic capacity of bovine granulosa cells on a per cell basis is higher than for ovine granulosa cells under similar culture conditions. Thirdly, cells from small bovine follicles attain levels of oestradiol secretion comparable to those of cells from large

follicles after 6 days in culture: however, this was not so for granulosa cells from small ovine follicles. Finally, IGF-I induces a maximal steroidogenic and proliferative response in bovine granulosa cells, whereas in ovine granulosa cells, the response can be augmented further by the addition of insulin and/or FSH. These differences in steroidogenic capacity between bovine and ovine granulosa cells could account for differences in the mechanism of dominance between the species (Campbell *et al.*, 1995).

In conclusion, we have developed a serum-free cell culture system for bovine granulosa cells which responds to physiological concentrations of FSH, IGF-I and insulin. This culture system will enable us to study the factors which regulate the physiological control of granulosa cell proliferation and differentiation, including structure: function relationships.

7. ULTRASTRUCTURAL CHARACTERISTICS OF BOVINE GRANULOSA CELLS ASSOCIATED WITH MAINTENANCE OF OESTRADIOL PRODUCTION *IN VITRO*

7.1 ABSTRACT.

The objective of the study was to determine if maintenance of oestradiol production by bovine granulosa cells *in vitro* was related to ultrastructural changes in the cells. The effect of including serum as an attachment factor on cell oestradiol production, morphology and ultrastructural characteristics was also analysed. Bovine granulosa cells from medium-sized follicles (4-8 mm) were cultured in a serum-free (SF) culture system or on serum-coated wells (SC). Medium was changed every 48 h for 6 days. After culture, cells were embedded for electron microscopy. SF-cultured cells maintained oestradiol production, but oestradiol secretion in SC cells declined with time in culture. The SF cells formed clumps consisting of two types of cells. Cells within the clumps were spherical and tightly joined to each other. The clumps were firmly attached to the plate by enlarged flattened cells. Ultrastructurally, the cells forming clumps had abundant rough and smooth endoplasmic reticulum (ER), and mitochondria with trabecular cristae. Gap junctions were extensive, and often had elongated pseudopodia which extended to contact other cells. Cells forming the base of the clumps and cells in SC wells were enlarged, contained less rough ER and fewer mitochondria which tended to be rounded. In addition, SC cells often contained endosome-like structures. In conclusion, granulosa cells cultured in serum-free conditions had ultrastructural characteristics that resembled those of granulosa cells *in vivo*. In contrast, granulosa cells growing either in the base of the clump, or grown as monolayers in serum-coated wells, had morphological characteristics suggestive of early luteinisation. These results may explain the granulosa cell loss of responsiveness in terms of oestradiol production and highlight the importance of structure: function relationship and cell: matrix interaction of granulosa cells.

7.2 INTRODUCTION

Tight regulation of follicular growth and development in cattle ensures that only the most competent follicle ovulates (Fortune *et al.*, 1991; Webb *et al.*, 1994; Campbell *et al.*, 1995). Growing follicles compete with other follicles, only one normally becoming dominant and progressing to final development. During this maturational process, granulosa cells increase expression of P₄₅₀ aromatase, P₄₅₀ side-chain-cleavage (Xu *et al.*, 1995a) and LH receptor mRNA (Xu *et al.*, 1995b). Ultrastructurally, granulosa cells in antral follicles contain mitochondria with parallel cristae, and the cytoplasm is rich in ribonucleoprotein particles (Bjersing, 1978). During luteinisation of granulosa cells, mitochondria acquire a rounded shape and cristae change to a tubular form, whilst the amount of smooth endoplasmic reticulum increases and rough endoplasmic reticulum decreases (McClellan *et al.* 1975). The mature dominant follicle is capable of ovulating in response to a gonadotrophin stimulus (Price and Webb, 1989), and forms a corpus luteum indistinguishable from a naturally-occurring corpus luteum (Schmitt *et al.*, 1996a), although progesterone production appears to be impaired (Rusbridge *et al.*, 1993). However, the morphological changes which accompany granulosa cell maturation and how these changes are controlled are largely unknown.

There have been numerous efforts to develop a physiologically relevant granulosa cell culture system for bovine granulosa cells. Unfortunately, granulosa cells in culture luteinise spontaneously, independently of gonadotrophins (Luck *et al.*, 1990). Thus detailed studies of the factors that regulate the development of aromatase activity in granulosa cells during follicle maturation have not been possible. The addition of serum during the first hours of culture, or pretreatment of culture plates with serum, is a common practice in granulosa cell culture studies, and facilitates cell adhesion to the plating surface (Kawate *et al.*, 1993; Wrathall and Knight, 1993; Gong *et al.*, 1994). Langhout *et al.* (1991) reported a 16-fold increase in granulosa cell number when cells were supplemented with serum during the first day of culture.

However, addition of serum does not prevent the decline in oestradiol production (Roberts and Echternkamp, 1994) and accelerates the increase in progesterone production (Luck *et al.*, 1990).

In the last Chapter (see Chapter 6) we described the development of a bovine granulosa cell culture system. In this system, granulosa cells produced significant amounts of oestradiol and remained responsive to physiological concentrations of FSH and growth factors. Instead of adopting the typical fibroblastic-like morphology of cells supplemented with serum, the granulosa cells became arranged into cell aggregates maintaining a spherical shape that resemble granulosa cells in the follicular wall. However, some fibroblastic-like cells which had attached to the culture well surface were evident at the base of these cell aggregates, anchoring the clumps to the culture surface as described previously for ovine cells (Campbell *et al.*, 1996a). Furthermore, treatment with high doses of FSH (or serum-coating of the culture well) increased the proportion of cells adopting this epithelial shape and this was associated with a reduction in oestradiol production (see Chapter 6). Since cell architecture and structure appear to be involved in cell signal transduction, and may determine the ability of the cells to respond to mitogenic factors (see Getzenberg *et al.*, 1990), culture systems which fail to preserve normal granulosa cell morphology may have limited physiological relevance.

The work of this Chapter analyses the morphological characteristics of undifferentiated bovine granulosa cells in a serum-free culture system which maintains oestradiol production and gonadotrophin responsiveness. The effects of inclusion of serum as an attachment factor on cell oestradiol production and morphological characteristics was also studied.

7.3 MATERIALS AND METHODS

7.3.1 GRANULOSA CELL CULTURE

Granulosa cells from medium-sized follicles (4-8 mm) were isolated and cultured in serum-free conditions as described in Chapter 2, with slight modifications. One

million viable granulosa cells per well were seeded in 6 well plates and cultured in serum-free (n= 3 experiments) conditions. Cells were incubated in 5 ml per well of culture medium for 144 h, replacing the medium completely every 48 h.

Alternatively, granulosa cells were cultured in monolayers (n = 3 experiments) under the same culture conditions, but in serum-coated wells. Briefly, culture wells were pre-coated with 2 ml of foetal donor serum and incubated overnight in a CO₂ incubator. Serum was then removed and the wells rinsed twice with 5 ml of culture medium. After 48 h of culture, serum-coated wells were flushed with 3 ml of culture medium to remove cells not attached to the culture surface to leave a cell monolayer, and cultured for a further 96 h. Culture medium was replaced completely every 48 h.

7.3.2 ULTRASTRUCTURAL STUDIES

Granulosa cells from medium-sized follicles were cultured for 144 h and prepared for electron microscopy. Oestradiol production from serum-free or serum-coated cells was measured in a single RIA and the intra-assay coefficient of variation was 6.5%.

7.4 RESULTS

7.4.1 CELL MORPHOLOGY

Granulosa cells were seeded as a homogeneous suspension of cells that initially covered the entire surface of the culture well. After 48 h in serum-free culture, cells formed aggregates in which two distinct types of cells were apparent. The cells forming the body of the clump had a spherical appearance similar to that of granulosa cells *in vivo* (Figure 7.1a), and were tightly joined. Cells localised at the bottom of the clump essentially formed the feet of the clump, attaching it to the culture surface. These cells were enlarged, flattened and fibroblastic-like (Figure 7.1a).

Cells cultured in serum-coated wells plated down, reaching confluence in the culture well. Cells which were growing over the cell layer attached to the culture surface

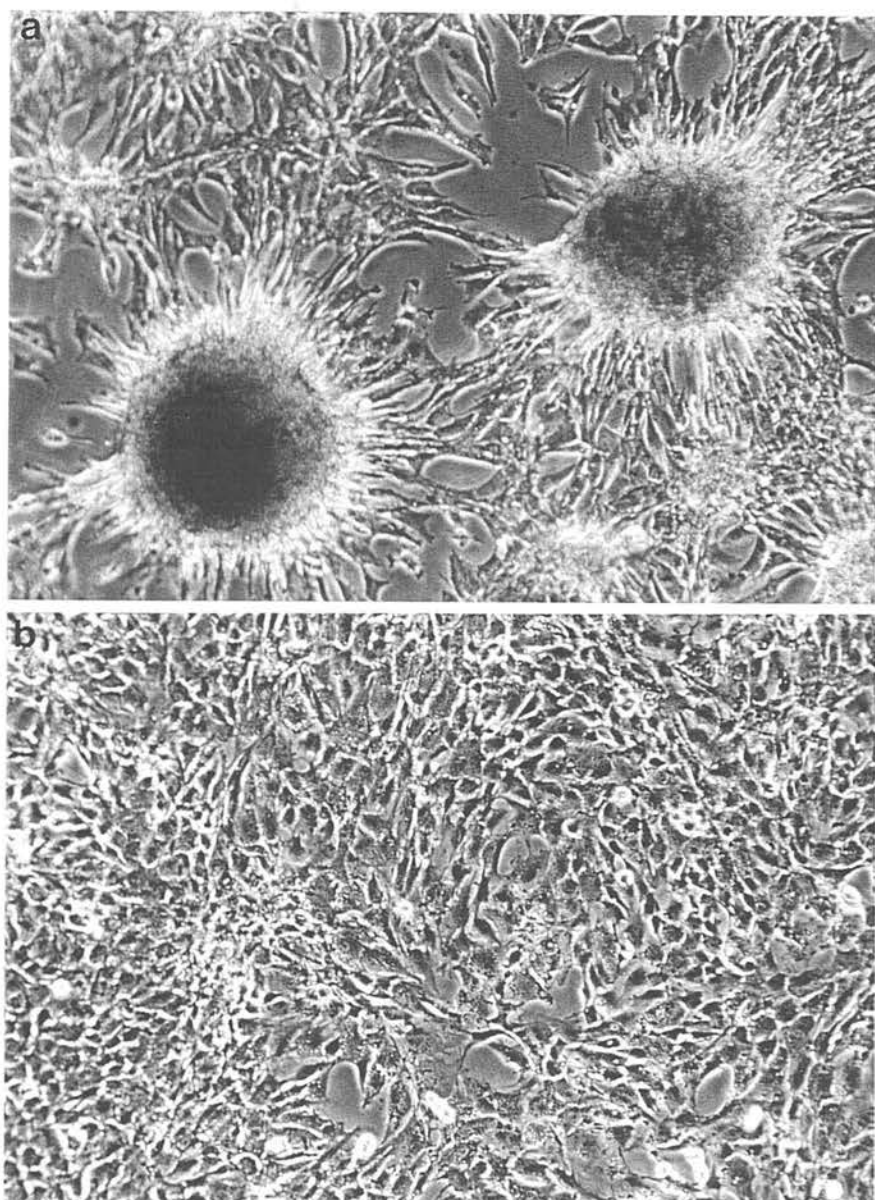


Figure 7.1. Bovine granulosa cells after 144 h of culture. Under serum-free conditions (a; X 100) granulosa cells formed cell aggregates (clumps) of spherical cells tightly bound together. Cells in contact with the plating surface differentiated in flattened fibroblastic-like cells that formed the feet of the clump. Granulosa cells cultured in serum-coated wells (b; X 100) plated down forming a cell monolayer that covered the entire surface of the well.

were removed by the flushing medium 24 h after the initiation of culture. The remaining cells formed a monolayer of fibroblastic-like cells with frequent cytoplasmic vacuolisations (Figure 7.1b).

7.4.2 OESTRADIOL PRODUCTION

Oestradiol production by granulosa cells in serum-free culture was maintained throughout the culture period (Figure 7.2). In contrast, oestradiol production by cells seeded in serum-coated wells declined ($p<0.01$) with time in culture (Figure 7.2), differing significantly ($p<0.01$) from the amount of oestradiol produced by cells in serum-free culture after 96 h.

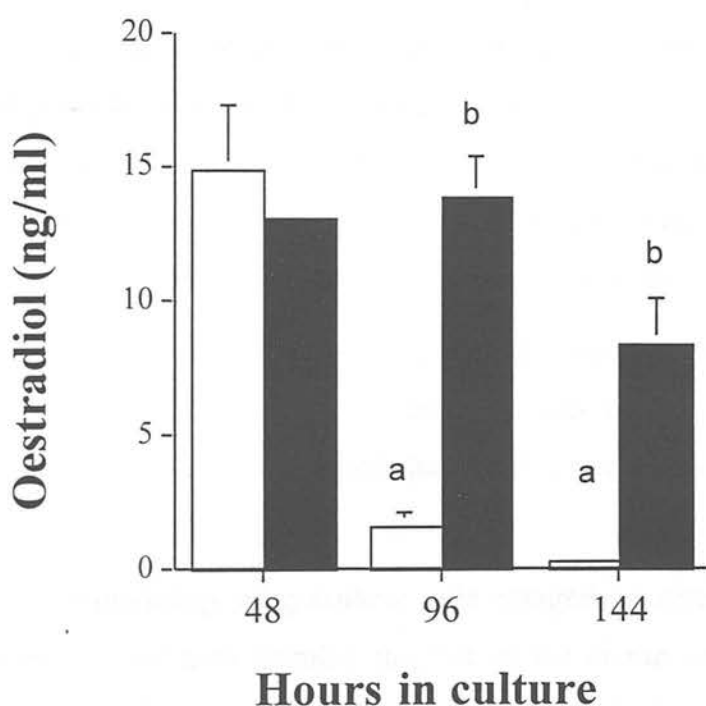


Figure 7.2. Oestradiol production by bovine granulosa cells after 0-48, 48-96 and 96-144 h of cultured, either in serum-free conditions (■), or in serum-coated wells (□). Values are means \pm SEM. Letters depict significant differences between treatments ($p<0.01$).

7.4.3 ULTRASTRUCTURAL CELL MORPHOLOGY

Granulosa cells cultured in serum-free conditions (Figure 7.3a) displayed characteristics similar to granulosa cells *in vivo*. Cells had a polyhedral shape, with a low cytoplasm to nuclear ratio, and were in close contact with each other. The cell membranes were closely apposed to each other, and attached through desmosomes and extensive gap junctions (Figure 7.3b). Contact between cells by microvillar projections in the intercellular spaces were also common. The main structures found in the cytoplasm of these cells were abundant smooth and rough endoplasmic reticulum, with dark central matrix, widely distributed throughout the cells and occasionally organised into multilaminar structures (Figure 7.3c). Mitochondria with trabecular cristae were also abundant. Other structures such as Golgi apparatus, lipid droplets and dense bodies were also seen. Occasionally, mitotic granulosa cells were observed, as well as apoptotic cell remnants which appeared either as highly circumscribed electron-dense masses internalised by neighbouring cells (apoptotic bodies; Figure 7.3a), or as apoptotic debris lying among cells (Figure 7.3d).

The cells forming the base of the clump (Figure 7.4a,b) were flattened, with a high cytoplasm/nuclear ratio. The endoplasmic reticulum was mainly of the smooth variety, and the mitochondria had a rounded shape with fewer cristae with a mixture of trabecular and tubular forms.

The ultrastructural morphology of granulosa cells cultured on serum-coated wells was similar to that of the cells forming the feet of the clump under serum-free conditions. However, cells cultured in serum-coated wells had strongly electron-dense cytoskeletal bundles running parallel to the plating surface (Figure 7.5b). These cells often contained large endosome-like structures (Figure 7.5a) filled with membrane-limited 'vacuoles'. However, these structures could not be identified as a specific type of organelle.

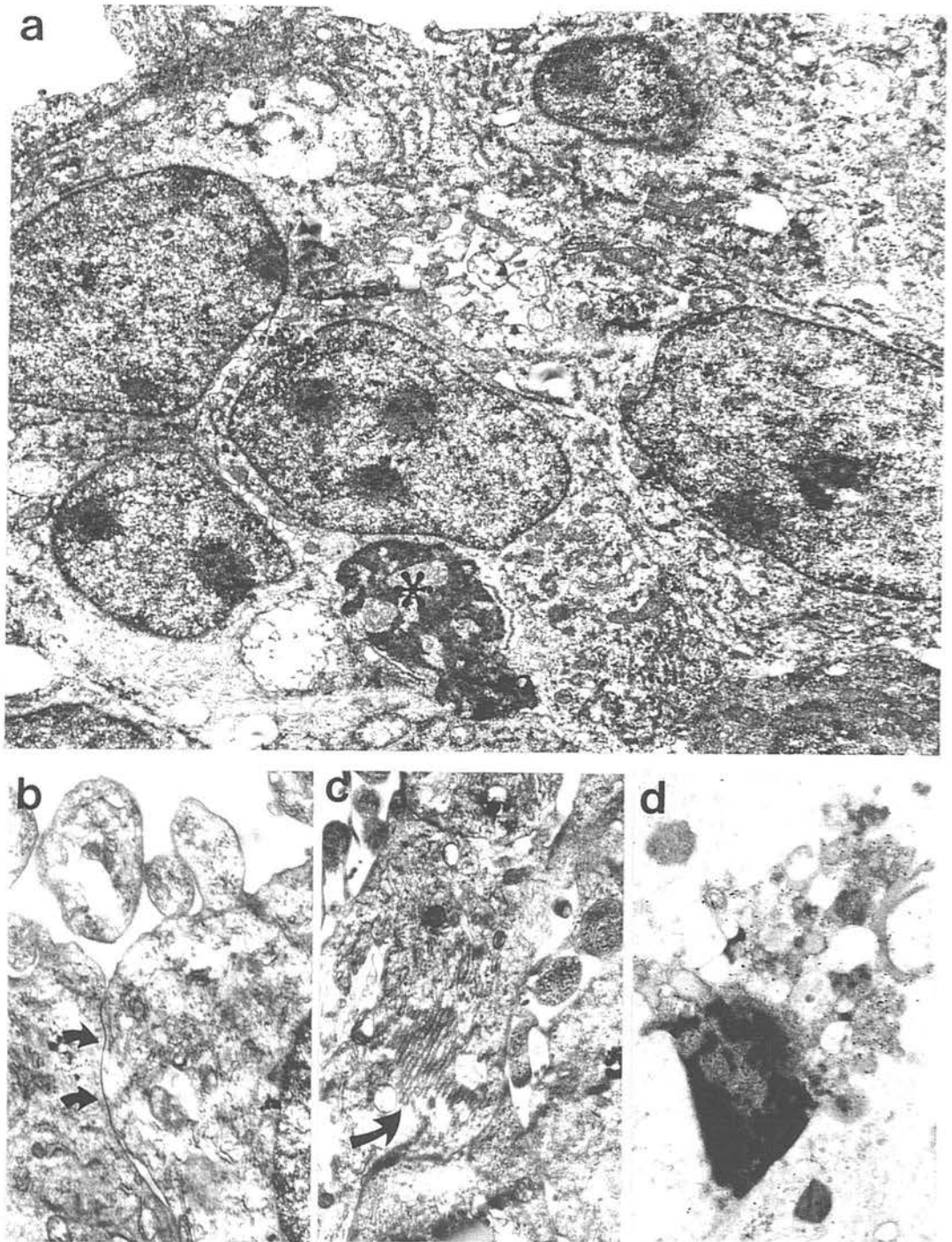


Figure 7.3. Ultrastructural morphology of serum-free cultured bovine granulosa cells. Within the clump (a; X 9102), polyhedral shaped cells were in close contact with each other. Cells possessed extensive gap junctions (b; X 14657) and abundant rough endoplasmic reticulum, occasionally organised in multilaminar structures (c; X 7614). Apoptotic remnants were found either internalised by neighbouring cells (a; *) or between them (d; X 9976).

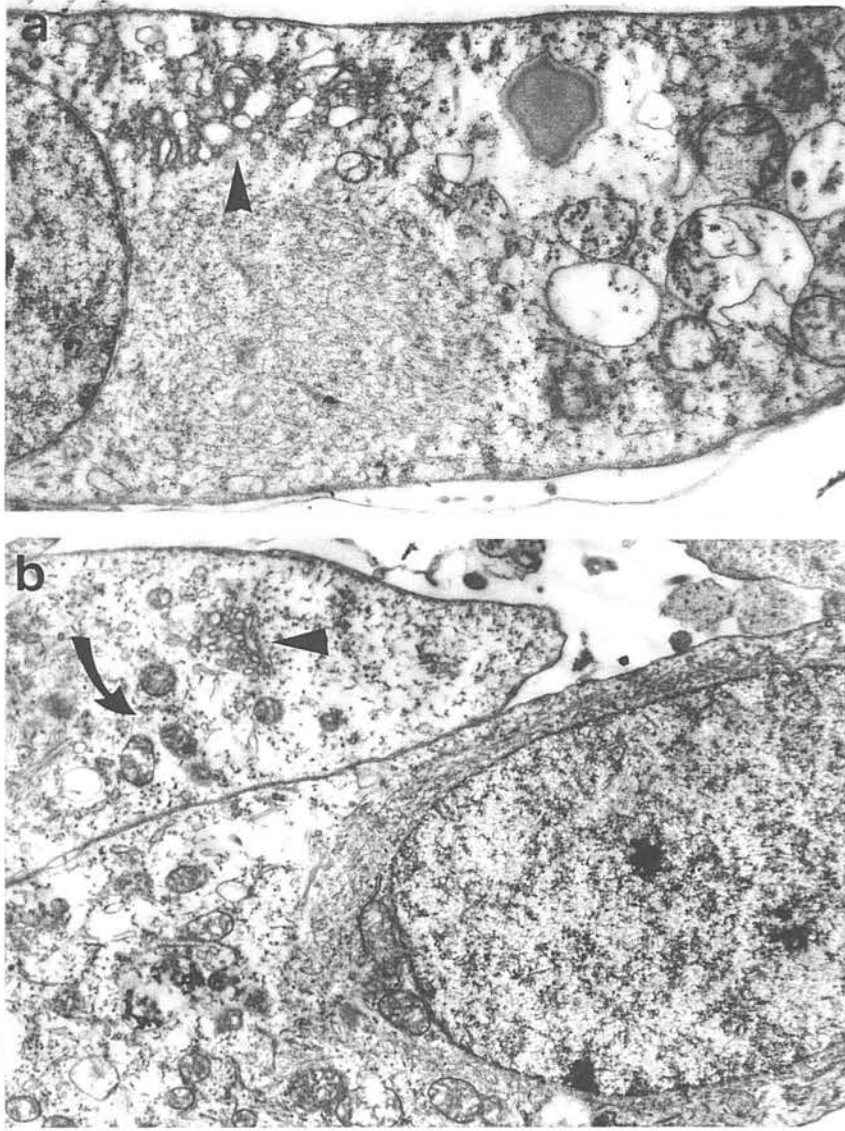


Figure 7.4. Ultrastructural morphology associated with cultured bovine granulosa cells that formed the feet of the cell clumps under serum-free conditions. These cells were flattened, with high cytoplasm/nuclear ratio and predominantly smooth endoplasmic reticulum (arrow head) and rounded mitochondria (long arrow). a; X 10440, b; X 7830.

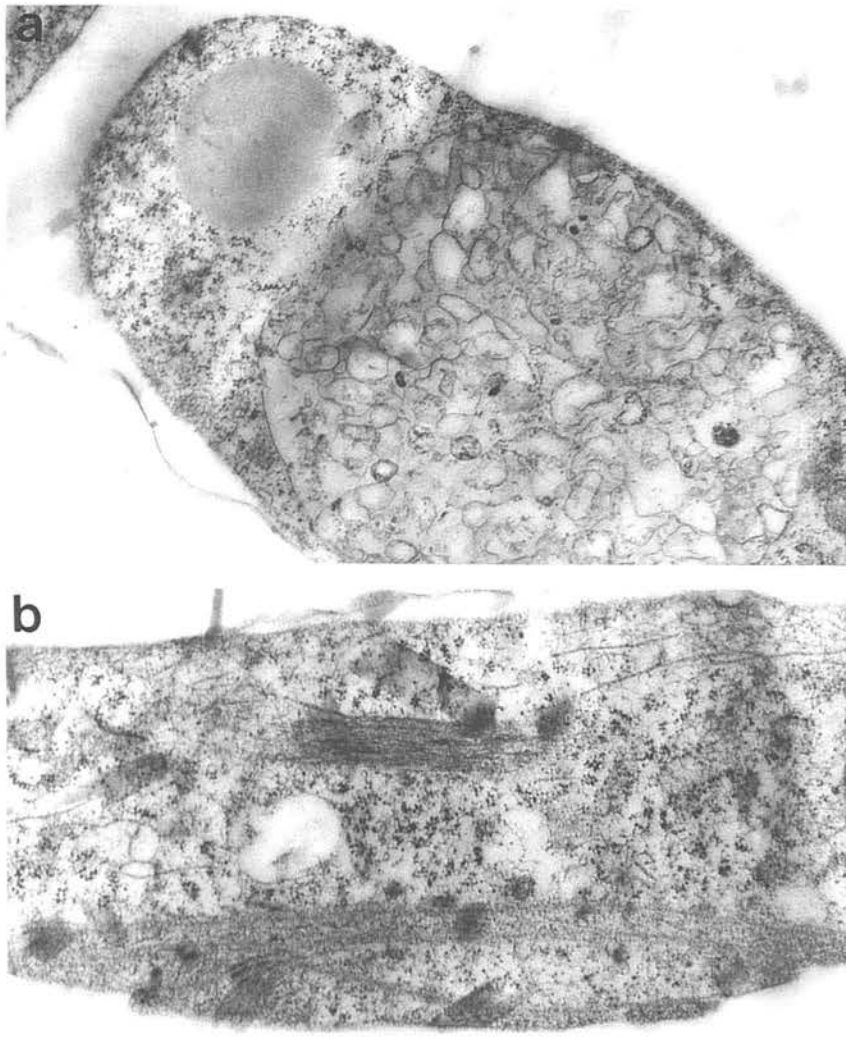


Figure 7.5. Electron micrograph of bovine granulosa cells cultured in serum-coated wells. Cells cultured in serum-coated wells had endosome-like structures (a; X 17897) and cytoskeletal bundles were visible running parallel to the plating surface (b; X 22902).

7.5 DISCUSSION

This Chapter has compared the ultrastructural characteristics of bovine granulosa cells cultured under conditions where normal cell function is either maintained or lost. The majority of cells cultured under serum-free conditions (where oestradiol production was maintained) retained normal morphology of granulosa cells seen *in vivo*, with abundant rough and smooth endoplasmic reticulum, mitochondria with trabecular cristae and extensive gap junctions. In contrast, the loss of aromatase activity in cells cultured on serum-coated wells was associated with an elongated cellular morphology, with rounded mitochondria and decreased abundance of rough endoplasmic reticulum, changes compatible with the onset of terminal differentiation and luteinisation.

Oestradiol production by granulosa cells seeded in serum-coated wells declined with time in culture, and had almost disappeared by 144 hours, similar to previous reports for cells cultured under similar conditions (Luck *et al.*, 1990; Langhout *et al.*, 1991, Gong *et al.*, 1994). It is possible that oestradiol-producing granulosa cells were inadvertently removed from the culture at 48 h, when wells were flushed with medium. However, cultured granulosa cells in serum-coated wells still produced oestradiol at 144 hours, although oestradiol produced by serum-free cultures was significantly higher than in serum-coated wells (see Chapter 6). As expected, granulosa cells in serum-free culture maintained their oestradiol production throughout the culture period.

Granulosa cells localised in the body of the clumps formed under serum-free conditions had characteristics similar to granulosa cells in the membrana granulosa of antral follicles (McClellan *et al.*, 1975; Bjersing, 1978) and maintained oestradiol secretion throughout the culture period. It is possible that clumping allows cell interactions with other granulosa cells, thus preventing their luteinisation. Similar associations between granulosa cell clumping and oestradiol production have been observed for ovine (Campbell *et al.* 1996a) and porcine (Picton *et al.*, 1994) granulosa cells cultured under serum-free conditions. Further evidence comes from the studies of Lavranos *et al.* (1994), in which bovine granulosa cells in an

anchorage-independent culture system formed cell aggregates that maintained non-luteinised granulosa cell ultrastructural characteristics, (although in this system the cells did not produce oestradiol despite being collected from 3-7 mm diameter antral follicles). Cell-cell interactions were mediated by gap-junctions in the granulosa cell membrane. Gap-junctions are abundant between granulosa cells in antral follicles, but are reduced in number after the LH surge *in vivo* (McCellan *et al.*, 1975) or following gonadotrophin stimulation *in vitro* (Sutovsky *et al.*, 1993). In the culture system studied here, gap-junctions were abundant between granulosa cells, suggesting that cell communication may play a role in the maintenance of aromatase activity and oestradiol production throughout the culture period. This was supported by observations that maintenance of aromatase activity in serum-free culture depended critically on the initial plating density (Campbell *et al.*, 1996a). Moreover, rat and porcine granulosa cell clusters in culture were shown to have increased numbers of LH receptors (Amsterdam and Rotmensch, 1987).

In contrast to granulosa cells growing within a clump, granulosa cells cultured as a monolayer on serum-coated wells showed a decline in oestradiol production with time. This decline in oestradiol production was not due solely to serum-coating of the plating surface as cells cultured in serum-coated wells continued to produce oestradiol throughout the culture period (see Chapter 6). In this study, the sharp decline in oestradiol may be a consequence of washing out cells which have maintained the granulosa cell phenotype, but which were not firmly attached to the plating surface. Oestradiol production by granulosa cells forming the feet of the clump in serum-free culture was not determined. However, their morphological characteristics were similar to cells cultured on serum-coated wells, suggesting that they had reduced aromatase activity. In both cases, cells had a fibroblastic-like appearance and displayed ultrastructural characteristics compatible with the onset of luteinisation. McClellan *et al.* (1975) described the changes that occur during the luteinisation of ovine granulosa cells after the LH surge. Luteinisation was characterised by gradually increasing amounts of smooth endoplasmic reticulum, a well-developed large Golgi system and rounding of mitochondria with tubular cristae (McClellan *et al.*, 1975; Bjersing, 1978; Amsterdam and Rotmensch, 1987). In

addition, changes observed during luteinisation correlated with the induction of steroidogenic enzymes in the mitochondria and endoplasmic reticulum of the cells (Rodgers *et al.*, 1996). However, the changes seen in the ultrastructure of organelles implicated in steroidogenesis were not specific to granulosa cells, as similar changes have been described for steroidogenic cells in the ovine foetal adrenal gland (Webb, 1980).

Fibroblastic-like cells were situated in contact with the plating surface, both in serum-free and serum-coated wells, suggesting that morphological luteinisation of granulosa cells occurs when cells make contact with structures other than granulosa cells themselves. Furthermore, when serum (Gong *et al.*, 1994; Langhout *et al.*, 1991) or components of the extracellular matrix (Saumande, 1991; Roberts and Echternkamp, 1994) are used to aid cell attachment, spontaneous luteinisation of granulosa cells occurs, emphasising the role of extracellular matrix components in granulosa cell differentiation (Amsterdam and Rotmensch, 1987; Luck, 1994). Indirect evidence for the role of the extracellular matrix on granulosa cell function comes from differences in oestradiol production of granulosa cells close to the basal lamina of the follicle compared to antral granulosa cells (Roberts and Echternkamp, 1994; Armstrong *et al.*, 1996a; Rouillier *et al.*, 1996).

Integrin receptors bind to components in the extracellular matrix, resulting in phosphorylation of intracellular proteins that play key roles in regulating the fundamental processes of growth and differentiation (Juliano, 1996). Furthermore, through their cytoplasmic domain, integrins interact with cytoskeletal proteins and other signalling molecules (Juliano, 1996) which can influence cell shape, alter gene expression and determine the response of the cell to specific ligands (Getzenberg *et al.*, 1990). Therefore, cell interactions with extracellular matrix may promote or maintain granulosa cell differentiation. Nonetheless, high doses of FSH (> 10 ng/ml) enhanced granulosa cell luteinisation in both serum- and extracellular matrix-free culture medium (see Chapter 6), although complete luteinisation of the whole population of cells was never achieved. Therefore, to devise a physiological culture system for the study of granulosa cell luteinisation, require consideration of the

interactions between extracellular matrix and gonadotrophins. The use of the culture model described herein could provide a valuable tool for the study of granulosa cell luteinisation *in vitro*.

Although observed only occasionally, the presence of apoptotic cell remnants in cultures indicates that cultured granulosa cells can undergo apoptosis. Apoptosis has been identified in the follicular wall *in vivo* (Hughes and Gorospe, 1991; Tilly, 1996). Furthermore, signs of apoptosis such as pyknosis (Logothetopoulos *et al.*, 1995) or DNA fragmentation (Jolly *et al.*, 1994) have been detected in healthy follicles. In the present study, apoptosis affected individual cells without affecting other healthy cells in the vicinity. Thus granulosa cell proliferation both *in vivo* and *in vitro* depends on a balance between cell division and cell death. Apoptotic bodies were observed which had been engulfed by neighbouring granulosa cells. Apoptotic bodies formed from dying cells within follicular walls *in vivo* or in cell clumps *in vitro*, may be engulfed by granulosa cells or infiltrating macrophages. However, apoptotic bodies may also undergo secondary autolysis (apoptotic necrosis; Majno and Joris, 1995), or shedding into the antral cavity or culture medium, particularly where cells are exposed to these fluids. These results are consistent with the hypothesis that granulosa cell death occurs by apoptosis.

In conclusion, using this cell culture system, in which cells in different stages of differentiation co-exist, the results of this Chapter extend the observations made in the previous Chapter (see Chapter 6) highlighting important structural characteristics in cells which maintain the granulosa cell phenotype and oestradiol production. Similarly, we have described characteristic ultrastructural changes that occur during differentiation and luteinisation *in vitro*. Further studies of the interactions between granulosa cells and components of the extracellular matrix (through adhesion receptors and cytoskeleton) might produce a better understanding of the mechanisms of luteinisation. Finally, culture of granulosa cells under serum-free conditions preserved granulosa cell oestradiol production, as well as their morphological and ultrastructural characteristics, whilst introduction of serum into the culture system

was associated with changes in ultrastructural characteristics and the loss of oestradiol production.

8. *IN VITRO* RESPONSES OF BOVINE GRANULOSA CELLS TO FSH, INSULIN AND IGF-I, AT DIFFERENT STAGES OF THE FIRST FOLLICULAR WAVE

8.1 ABSTRACT.

The objectives of this study were to determine the effect of the dominant follicle *in vivo* on granulosa cell proliferation and oestradiol production *in vitro*. After synchronisation of oestrus, granulosa cells were isolated from small (<4 mm), medium-sized (4-8 mm) and large (>8 mm) follicles collected either on day 3 or day 7 of the oestrous cycle. The effects of FSH, insulin and IGF-I were then evaluated in culture. Granulosa cells from day 7 medium-sized follicles secreted significantly ($p<0.01$) less oestradiol than cells from day 3 medium-sized follicles during the first 48 h of culture. However, oestradiol production from small and large follicles did not vary ($p>0.05$). After long-term culture (144 h), there were no differences ($p>0.05$) in oestradiol production of cells from day 3 or day 7 follicles and cells of all three size categories responded similarly to stimulation with FSH, insulin and IGF-I. However, the proliferative response of the cells to FSH stimulation varied ($p<0.05$) with the day of the oestrous cycle. While granulosa cells from day 3 small and medium-sized follicles exhibited proliferative responses to FSH, cells from day 7 follicles did not exhibit this response. This difference in proliferative response in granulosa cells from large follicles obtained on day 3, or day 7, was also observed with cells stimulated with IGF-I. These results show that both oestradiol production and proliferative capacity of granulosa cells from medium-sized follicles *in vitro* were affected by the stage of the follicular wave, *i.e.* by the presence of a dominant follicle during their development *in vivo*. After long-term culture, oestradiol production did not differ between granulosa cells from day 3 or day 7 follicles, but there were differences in proliferative responses to FSH, insulin and IGF-I according to follicle size and stage of the follicular wave.

8.2 INTRODUCTION

Ovarian follicular growth in cattle occurs in a wave-like pattern (Sirois and Fortune, 1988; Roche and Boland, 1991; Savio *et al.*, 1993). Under the same hormonal milieu, a cohort of follicles begins its growth simultaneously, until one follicle is selected for final maturation. This follicle appears to be involved in a mechanism that inhibits the growth of the remaining gonadotrophin-dependent follicles that become atretic, a process known as 'dominance' (Fortune *et al.*, 1991). The dominant follicle is capable of ovulation (Price and Webb, 1989), but if there is no ovulatory LH surge, it becomes atretic and a new wave of follicles grow. In cattle, there are characteristically two or three follicular waves per oestrous cycle (Sirois and Fortune, 1988; Adams *et al.*, 1993; Gong *et al.*, 1995). The first follicular wave is detected on day 2-3 of the oestrous cycle and the dominant follicle is fully developed by day 7.

The mechanism by which the selected follicle achieves dominance over other follicles in the cohort is still not fully understood, but must include the suppression of follicle development in both ovaries (Webb *et al.*, 1992). Follicular dominance may be exerted either indirectly, by the negative feedback influence on pituitary FSH secretion, and/or by a direct mechanism mediated through a factor secreted by the dominant follicle that inhibits follicular development of the subordinate follicles in both ovaries.

Recruitment and growth of follicles is primarily dependent on FSH (McNeilly *et al.*, 1991, Gong *et al.*, 1996a) and a peak of FSH precedes the emergence of each wave of follicles (Adams *et al.*, 1993; Gong *et al.*, 1995; see Chapter 4). The dominant follicle secretes factors such as oestradiol and inhibin capable of suppressing the release of FSH by the pituitary gland (Baird *et al.*, 1991). For example, bovine follicular fluid treated with charcoal to remove steroids, but containing inhibin, can suppress the postovulatory peak of FSH, and delay the emergence of the follicular wave (see Fortune, 1994). During the follicular phase, follicular fluid can halt the growth of the dominant follicle and retard the return to oestrus after luteolysis in cattle (Turzillo and Fortune, 1993).

Ovarian production of factors other than inhibin that suppress follicular development have been demonstrated. For example, steroid- and inhibin-free follicular fluid inhibited follicular growth and delayed the return to oestrus in heifers (Law *et al.*, 1992; Wood *et al.*, 1993) and ewes (Campbell *et al.*, 1991). Similarly, Campbell and Scaramuzzi (1996) found a decrease in the ovarian antral follicle population and steroid production in sheep after the intraovarian infusion of steroid-free follicular fluid, without affecting circulating FSH concentrations. The nature of this factor(s), and its mechanism of action, has not been elucidated. However, *in vitro* studies have recently demonstrated that inhibin-free follicular fluid fractions reduce oestradiol production and cell proliferation of sheep granulosa cells (Baxter *et al.*, 1995).

It has been demonstrated that the presence of a dominant follicle has a negative effect on the number of follicles that respond to a gonadotrophic superovulatory treatment (Guibault *et al.*, 1991; Adams *et al.*, 1993). Thus, bovine granulosa cells that have been under the influence of a dominant follicle may differ in their steroidogenic capacity or response to FSH, compared to cells that have not been exposed to factors secreted by the dominant follicle. In Chapter 6, it was observed that treatments with FSH, insulin and IGF-I increased oestradiol production and proliferation of bovine granulosa cells *in vitro*. Furthermore, high insulin concentration *in vivo* (see Chapter 4) was associated with increased follicular recruitment. It has been proposed that the increased follicle number associated with enhanced feed intake is the result of rescuing follicles from atresia (sheep, Downing and Scaramuzzi, 1991; pigs, Maurasse *et al.*, 1985; Matamoros *et al.*, 1990; 1991). In this Chapter, the effect of the presence of the dominant follicle on the steroidogenic capacity of cultured bovine granulosa cells *in vitro* in response to physiological levels of FSH, insulin, and IGF-I was assessed with cells from follicles at two stages of the oestrous cycle, *i.e.* day 3 (prior to selection of the dominant follicle; Sunderland *et al.*, 1994), and day 7 (when a dominant follicle is present in the ovaries).

8.3 MATERIALS AND METHODS

8.3.1 EXPERIMENTAL ANIMALS.

The oestrous cycles of non-lactating Friesian cows were synchronised (see Chapter 2). Only animals observed in oestrus between 48 and 72 h after implant withdrawal were used.

On the afternoon before the collection of the ovaries on either day 3 or day 7 of the oestrous cycle, the stage of the oestrous cycle was determined and the diameter of the largest follicles measured by ultrasound. Next day animals were slaughtered and the ovaries placed in collection media at 37°C, as described previously (see Chapter 2), before transportation to the laboratory for follicle dissection and granulosa cell isolation and culture. Granulosa cell culture was performed as described in Chapter 2. For each follicle size category and day of the oestrous cycle, between 40 to 75 x 10³ viable cells per well were seeded in 96 well plates and oestradiol production and cell number estimated after culture as described in Chapter 2.

8.3.2 EXPERIMENT 1.

A total of sixteen cows were synchronised in two replicate experiments. Two animals were excluded because one did not show oestrus within the expected time and the other developed a follicular cyst. From the remaining fourteen animals, granulosa cells from small, medium-sized and large follicles of ovaries collected either on day 3 (n = 9) or day 7 (n = 5) of the oestrous cycle were cultured, and the effects of insulin (0-5000 ng ml⁻¹) and FSH (0-50 ng ml⁻¹; NIDDK-oFSH-S16) on cell number and oestradiol production were tested in a 6 x 4 factorial design, with four replicates per treatment.

8.3.3 EXPERIMENT 2.

A total of eighteen Friesian cows were used in two experiments. Granulosa cells from small, medium-sized and large follicles were collected either on day 3 (n = 9) or day 7 (n = 9) of the oestrous cycle. Cells were cultured with 1 ng ml⁻¹ of bFSH-I2, and the effects of insulin (10 ng ml⁻¹), LR3-IGF-I (10 ng ml⁻¹) or the combination of both insulin (10 ng ml⁻¹) and LR3-IGF-I (10 ng ml⁻¹) were tested on granulosa cell

number and oestradiol production. On the second occasion, day 3 animals had insufficient large follicles to obtain a representative pool of cells, and therefore this group contains data from the first culture only.

8.3.4 RADIOIMMUNOASSAYS

Oestradiol in culture media was measured by RIA with inter- and intra-assay coefficients of variation of 6.5% and 8.8% respectively. Plasma progesterone concentrations were measured by RIA in a single assay. The intra-assay coefficient of variation was 3.9%.

8.3.5 STATISTICAL ANALYSES

The effects of day of the oestrous cycle, time of culture, and effects of FSH, insulin and IGF-I were analysed by ANOVA, allowing for the variation of culture, pool of cells and well (random effects). Oestradiol production was analysed after logarithmic transformation of oestradiol production $\ln(E_2+1)/24 \text{ h}/10^4 \text{ cells}$. At 48 h, oestradiol production was calculated on the basis of the number of cells seeded. At 144 h, oestradiol production was calculated on the basis of the number of viable cells at the end of culture. Cell number at the end of culture was analysed by inclusion of the number of cells seeded as a covariant in the analysis. The plating densities for experiments 1 and 2 were 65 ± 1 and 64.5 ± 1 thousand cells per well respectively.

8.4 RESULTS

Animals on day 3 of the oestrous cycle had an immature corpus luteum (CL) weighing only $0.81 \pm 0.28 \text{ g}$. In contrast, animals on day 7 of the oestrous cycle had a well-developed CL, weighing $3.81 \pm 0.28 \text{ g}$. This was reflected in plasma progesterone concentrations (0.26 ± 0.16 and $1.06 \pm 0.16 \text{ ng ml}^{-1}$ for day 3 and 7 respectively; $p < 0.05$). The diameter of the largest follicle as measured by ultrasonography 14 h before the animals were slaughtered was $0.70 \pm 0.11 \text{ cm}$ for animals on day 3 and $1.50 \pm 0.11 \text{ cm}$ for day 7 ($p < 0.01$).

8.4.1 EXPERIMENT 1.

8.4.1.1 *Oestradiol*. After 48 h of culture, granulosa cell oestradiol production was lowest for cells from small follicles ($p < 0.01$), intermediate for cells from medium-sized follicles and highest for cells from large follicles (Figure 8.1a). There was no difference in oestradiol production by granulosa cells whether removed from follicles on day 3 and day 7 of the oestrous cycle in either the small or large follicle categories. However, oestradiol secretion by granulosa cells from medium-sized follicles differed significantly ($p < 0.01$) with the day of oestrous cycle. Cells from day 3 animals produced significantly higher amounts of oestradiol than cells from animals on day 7 of the oestrous cycle (Figure 8.1a). However, by 144h of culture, oestradiol production by granulosa cells from follicles removed either on day 3 or day 7 of the oestrous cycle was similar ($p > 0.05$) for all three size categories (Figure 8.1b).

After 6 days of culture, oestradiol production by granulosa cells from all three size categories responded to insulin and FSH in a dose-responsive manner (Figure 8.2). However, there were no significant differences in overall oestradiol production ($p > 0.05$), or responses to insulin or FSH (day \times insulin and day \times FSH interaction; $p > 0.05$) for granulosa cells removed either on day 3 or 7 of the oestrous cycle. However, oestradiol secretion in response to FSH stimulation varied ($P < 0.01$) with the dose of insulin (Figure 8.2). Without insulin, FSH inhibited ($P < 0.05$) oestradiol production by cells from small and medium-sized (Figure 8.2a and b) follicles. Overall, physiological doses of insulin (0.5 and 5 ng ml^{-1}) stimulated granulosa cell oestradiol production ($p < 0.01$), and this was augmented by 5 ng ml^{-1} FSH ($p < 0.01$) in all three follicle size categories. However, this stimulation was lost at higher FSH concentrations (50 ng ml^{-1}). Supraphysiological doses of insulin ($> 50 \text{ ng ml}^{-1}$) alone also stimulated oestradiol production. The addition of FSH to cells cultured with supraphysiological concentrations of insulin failed to augment oestradiol secretion, and became inhibitory at doses greater than 5 ng ml^{-1} ($p < 0.01$), in all three size categories.

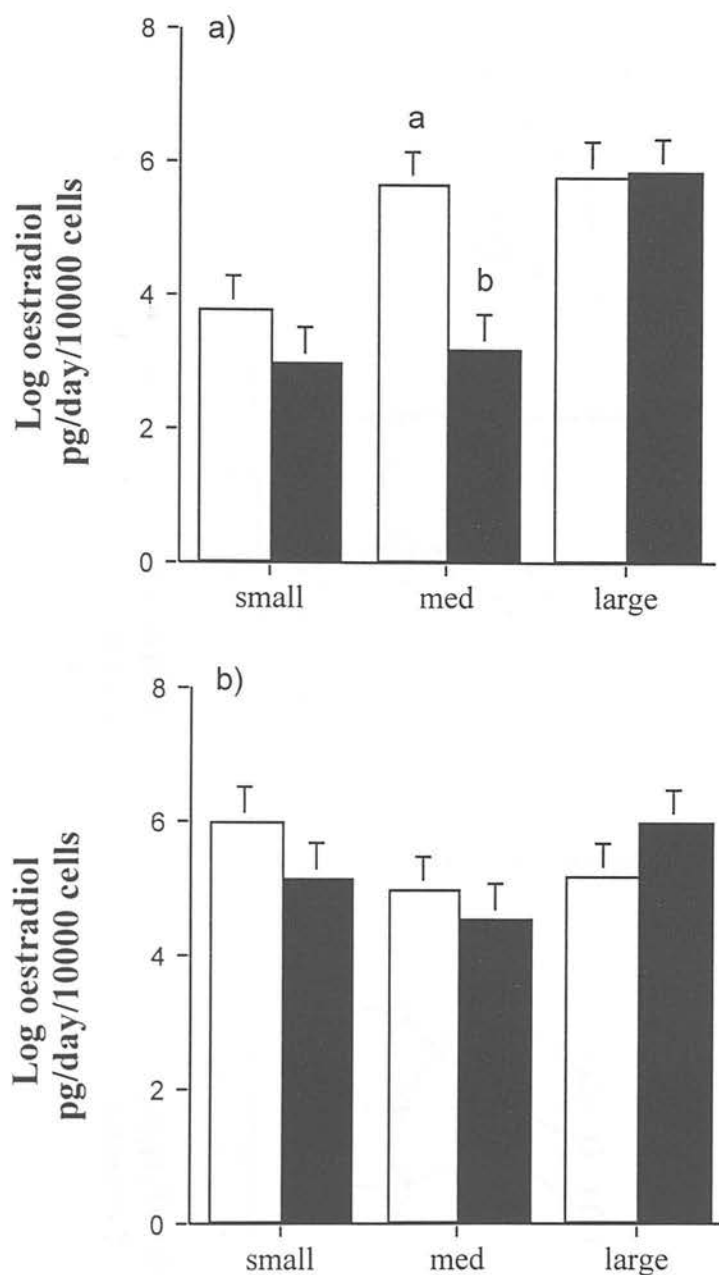


Figure 8.1. Oestradiol production per 10,000 bovine granulosa cells from small (<4 mm), medium-sized (4-8 mm) and large (>8 mm) follicles removed from ovaries on day 3 (□) or day 7 (■) of the oestrous cycle after 0-48 (a), and 96-144 h (b) of culture with 5 ng ml⁻¹ insulin and 5 ng ml⁻¹ oFSH-S16. Values are least square means \pm SEM. a, b indicate significant difference ($p < 0.01$) within the same follicle-size category.

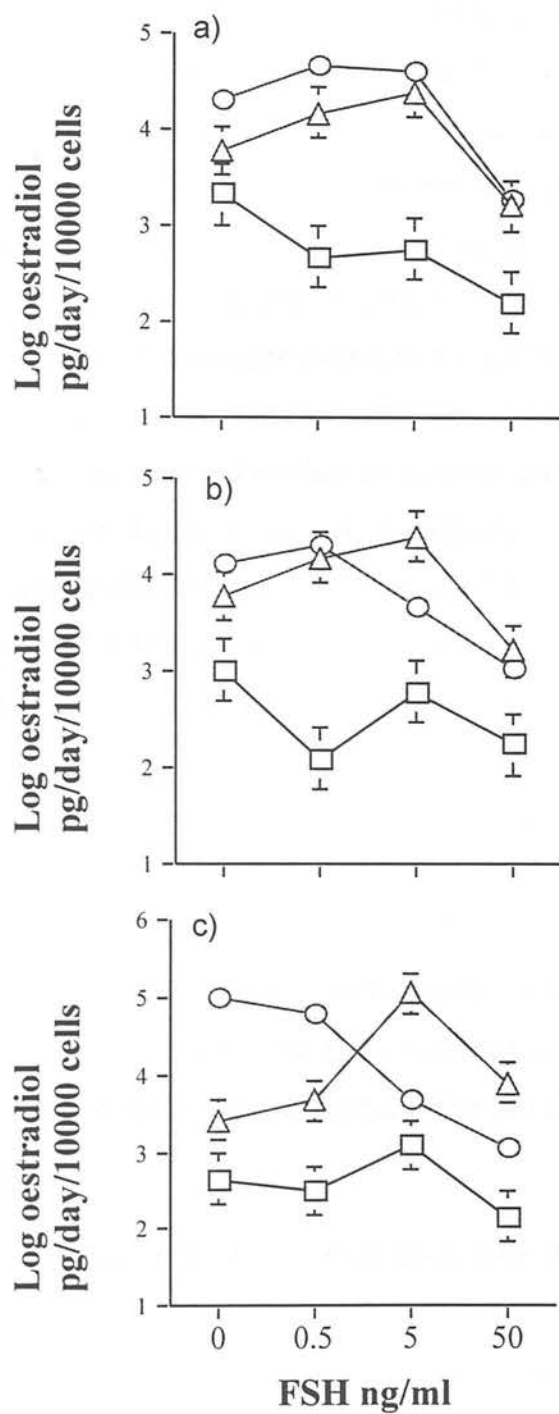


Figure 8.2. Effects of FSH on oestradiol production per 10,000 bovine granulosa cells from small (a), medium-sized (b) and large (c) follicles after culture for 144 either without insulin (□), with physiological (0.5 and 5 ng ml⁻¹) doses of insulin (Δ) or with supraphysiological (50, 500 and 5000 ng ml⁻¹) doses of insulin (○). Values are least square means ± SEM.

8.4.1.2. *Cell number.* In contrast to oestradiol production, granulosa cell number at the end of culture varied in response to insulin and FSH (Figure 8.3) depending on the day of the oestrous cycle when granulosa cells were harvested (day x insulin and day x FSH interaction; $p < 0.05$). Overall, insulin treatment increased granulosa cell number in a dose-responsive manner ($p < 0.01$) up to 50 ng ml^{-1} for cells recovered on day 3 and day 7 of the oestrous cycle. However, cells from medium-sized follicles on day 3 showed a greater response to insulin stimulation than their day 7 counterparts ($p < 0.01$). Cell proliferation in response to FSH varied according to both the concentration of insulin in culture, and the day of oestrous cycle that the cells were harvested. Cells from small (Figure 8.3a) and medium-sized (Figure 8.3b) follicles from day 3 of the oestrous cycle, (but not day 7, Figure 8.3d, e), increased in number in response to FSH (day x FSH interaction; $p < 0.05$). Without insulin, FSH increased ($p < 0.01$) cell numbers in small (Figure 8.3a) and medium-sized (Figure 8.3b) follicles cultured from day 3, but not ($p > 0.05$) day 7 (Figure 8.3d, e), nor in granulosa cells from large follicles (Figure 8.3c, f). Physiological doses of insulin (0.5 or 5 ng ml^{-1}) increased ($p < 0.05$) cell number *in vitro* in cells from either small (Figure 8.3a) or medium-sized (Figure 8.3b) day 3 follicles. Moreover, high doses of FSH (50 ng ml^{-1}) stimulated ($p < 0.05$) cell number (Figure 8.3a, b). At supraphysiological doses of insulin (50 ng ml^{-1} or above), cell number increased ($p < 0.01$) further, but addition of FSH had no further effect on cell number ($p > 0.05$).

8.4.2 EXPERIMENT 2.

8.4.2.1. *Oestradiol.* Granulosa cell oestradiol production after 48 h of culture (Figure 8.4) was directly related to follicle size, with oestradiol production increasing ($p < 0.01$) with increasing follicle diameter. Within each size category, oestradiol secretion by cells cultured in the presence of insulin, IGF-I or a combination of both did not differ ($p > 0.05$). Oestradiol production by granulosa cells from medium-sized follicles removed on day 3 of the oestrous cycle differed significantly ($p < 0.01$) from that of day 7 follicles at 48 h. However, after 144 h of culture (Figure 8.4), this difference was no longer evident ($p > 0.05$). Oestradiol production by granulosa cells from day 7 small and medium-sized follicles increased with time in culture ($p < 0.01$).

In contrast, oestradiol production by granulosa cells from day 3 medium-sized follicles was only maintained ($p>0.05$), whereas oestradiol production by granulosa cells from both day 3 and day 7 large follicles decreased ($p<0.01$). Finally, comparing within-day and between-size categories, there was no effect of insulin, IGF-I or a combination of both hormones on oestradiol production ($p>0.05$).

8.4.2.2. Cell number. Granulosa cell number after 144h of culture (Figure 8.5) did not differ significantly (SEM between days=50; $p>0.05$) for cells removed from follicles on either day 3 or day 7 of the oestrous cycle. However, for cells from large day 3 follicles, IGF-I, alone or in combination with insulin, increased cell number ($p<0.01$) compared to cells treated with insulin only. The response of granulosa cells from small and medium-sized follicles, to insulin, IGF-I or a combination of both did not vary significantly (day by treatment interaction; $p>0.05$). However, granulosa cells from large follicles removed on day 3 of the oestrous cycle responded to IGF-I with significantly greater ($p<0.01$) proliferation than granulosa cells from large follicles removed on day 7 of the oestrous cycle.

Figure 8.3. Effects of FSH on bovine granulosa cell number after 144 h of culture, from small (a), medium-sized (b) and large (c) follicles removed on day 3 of the oestrous cycle and small (d), medium-sized (e) and large (f) follicles removed on day 7 of the oestrous cycle, cultured without insulin (\square), physiological (0.5 and 5 ng ml⁻¹) doses of insulin (\triangle) or supraphysiological (50, 500 and 5000 ng ml⁻¹) doses of insulin (\circ). Values are least square means \pm SEM adjusted for the number of cells seeded as a covariate.

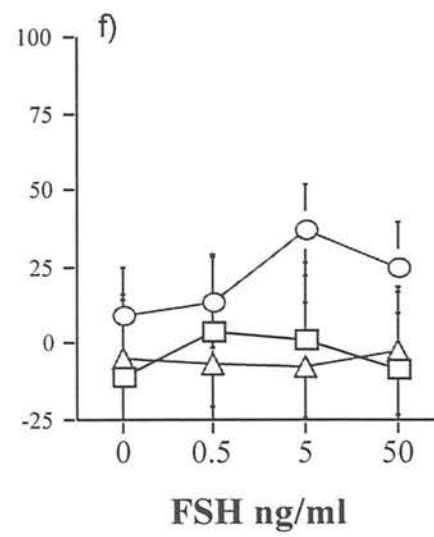
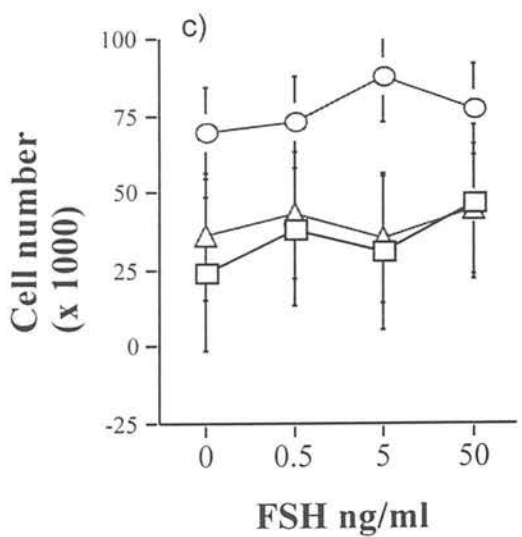
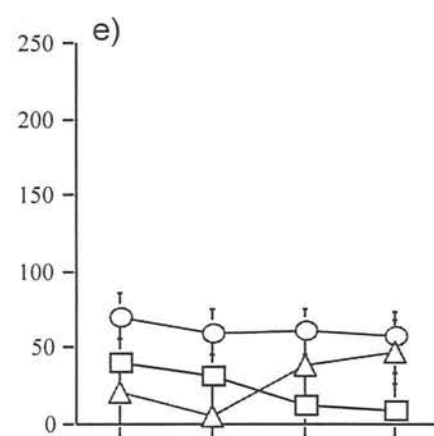
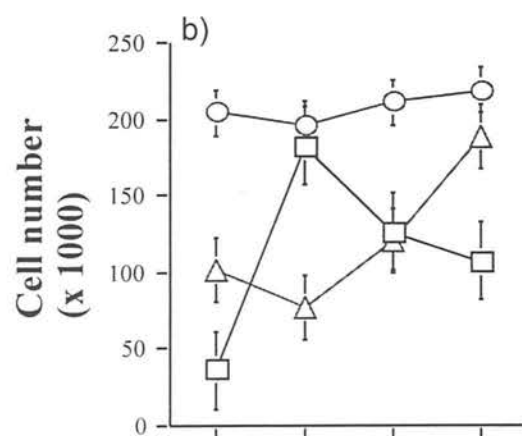
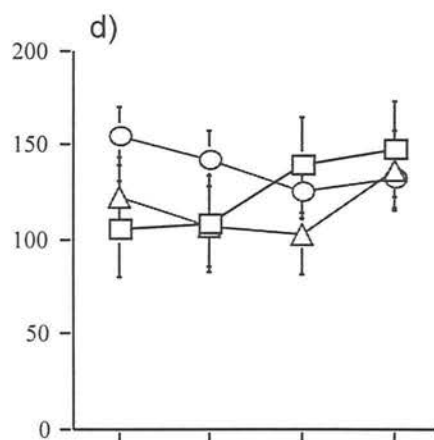
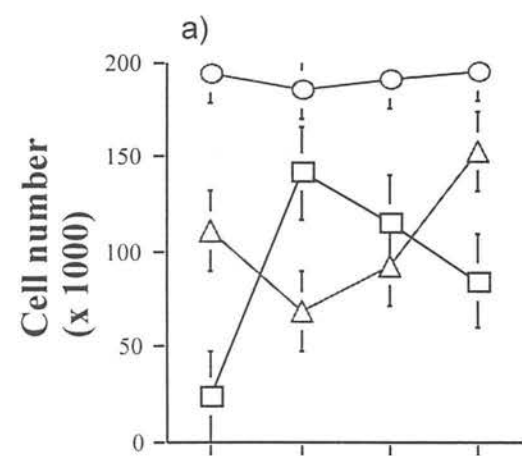


Figure 8.4. Effects of day of the oestrous cycle on oestradiol production per 10,000 bovine granulosa cells from small (a), medium-sized (b) and large (c) follicles removed on day 3 (□) and day 7 (■) of the oestrous cycle after 0-48, and 96-144 h in culture. Values are least square means \pm SEM. a, b indicate a significant difference ($p < 0.01$) within the same follicle-size category.

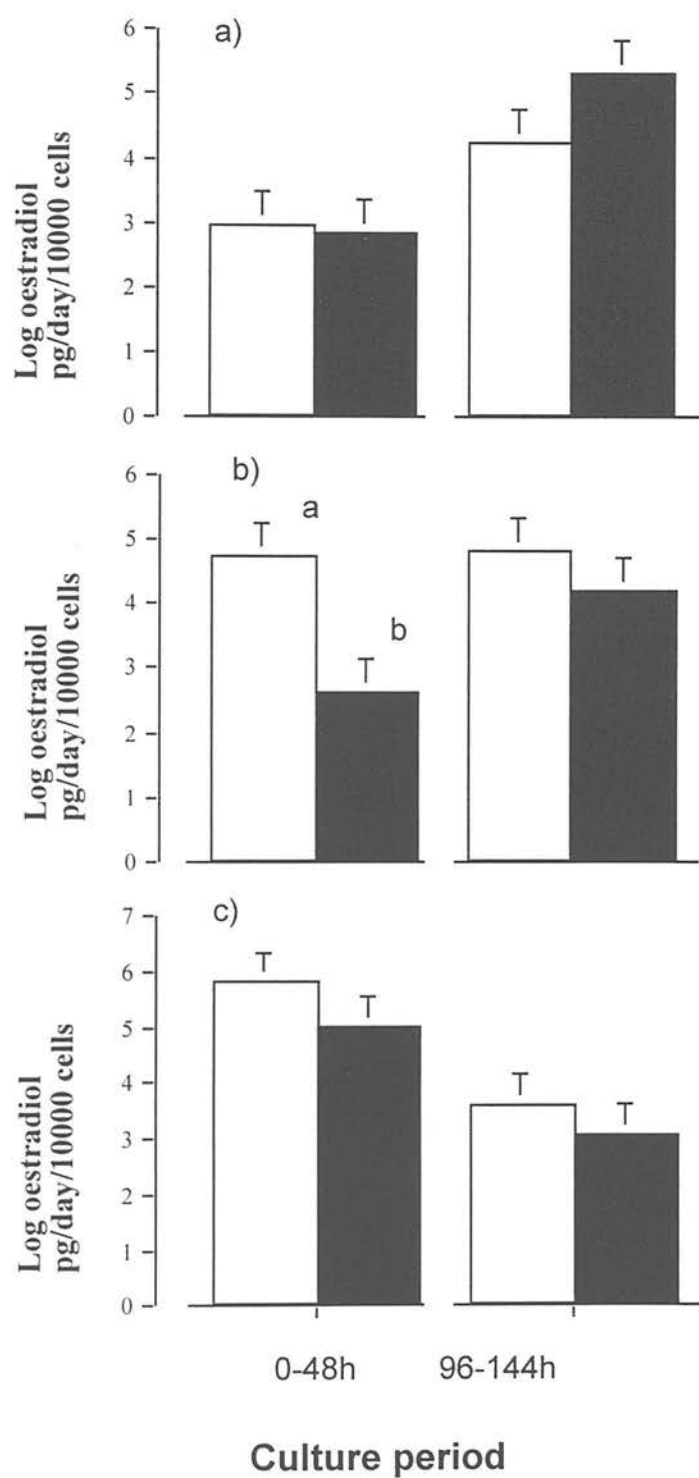
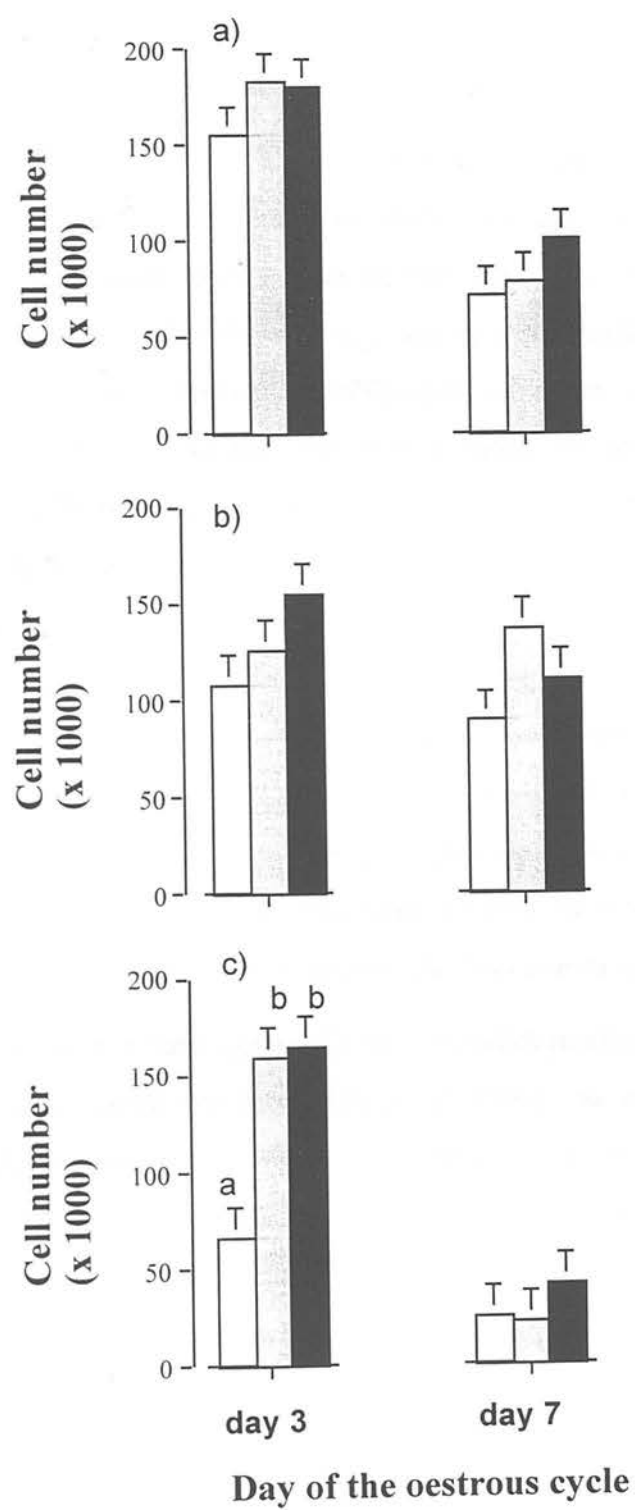


Figure 8.5. The effects of either 10 ng ml⁻¹ insulin (□), 10 ng ml⁻¹ LR3-IGF-I (□) or insulin plus LR3-IGF-I (■) on the number of bovine granulosa cells after 144 h of culture from small (a), medium-sized (b) and large (c) follicles removed either on day 3 or day 7 of the oestrous cycle. All cultures were in the presence of 1 ng ml⁻¹ of FSH. a, b indicate a significant difference (p<0.01) within the same day and follicle size category.



8.5 DISCUSSION

These studies demonstrated that the initial steroidogenic and proliferative capacity of bovine granulosa cells *in vitro* differed according to both follicle size and the stage of the follicular wave at which the ovaries were removed.

Specifically, granulosa cells from subordinate medium-sized follicles removed on day 7 of the oestrous cycle had reduced ($p < 0.01$) oestradiol production during the first 48 h of culture compared to cells from medium-sized follicles removed on day 3 of the oestrous cycle (*i.e.* before the establishment of a dominant follicle). However, this difference in oestradiol production disappeared with time in culture (144 h). In contrast to cells removed from medium-sized follicles, the aromatase activity of bovine granulosa cells from either small or large follicles did not differ with day of the oestrous cycle or time in culture. Granulosa cells from the three follicle size categories removed either on day 3 or 7 of the oestrous cycle secreted similar amounts of oestradiol in response to FSH stimulation during the final period of culture (96-144 h). However, there was a differential proliferative response to FSH, with cells from day 3 small (Figure 8.3a) and medium-sized (Figure 8.3b) follicles proliferating in response to FSH, whereas cells from day 7 follicles did not (Figure 8.3d,e). These results suggest that medium-sized follicles are more susceptible to the changes in the hormonal milieu that occur after the selection of the dominant follicle.

In immature follicles (< 4 mm), granulosa cell oestradiol production (see Chapter 6) and mRNA P450-aromatase expression (Xu *et al.*, 1995a) are consistently low, and both increase after the follicle reaches 4 mm in diameter. On the other hand, the lack of difference of stage of the oestrous cycle on oestradiol production by cells from mature follicles (> 8 mm) in this study agrees with the results of Tian *et al.* (1995). They found that, although oestradiol concentrations in follicular fluid of the dominant follicle increased after luteolysis, expression of P450 aromatase did not increase. Thus, the increase in oestradiol production by the preovulatory follicle may be due to increased production of aromatisable substrate by theca cells. Consequently, large follicles from day 3 may have already reached a stage of maturity, in terms of aromatase activity, similar to that of large follicles on day 7 of

the oestrous cycle. Additional support for this concept is the observation that oestradiol production by granulosa cells from large follicles cannot be stimulated by FSH *in vitro* (Berndston *et al.*, 1995; see Chapter 6).

It is interesting that differences in oestradiol production by granulosa cells removed on day 3 and day 7 of the oestrous cycle were observed in medium-sized follicles. Expression of mRNA P450-aromatase increased in follicles within this size range (4–8 mm) (Xu *et al.*, 1995a). Further, LH receptor mRNA expression in granulosa cells appeared at around 8 mm in diameter (Xu *et al.*, 1995b), indicating further differentiation of the granulosa cells. It is also in follicles of this size-category that dominant follicle selection occurs and the highest proportion of antral follicles undergo atresia (Fortune, 1994). Xu *et al.* (1995a) reported a reduction in expression of mRNA for the steroidogenic enzymes, P450 aromatase and P450_{scc}, and reduced expression of mRNA for FSH receptor (Xu *et al.*, 1995b) in granulosa cells of follicles undergoing atresia. Similarly, subordinate bovine follicles have follicular fluid oestradiol to progesterone ratios below 1, indicating a reduction in aromatase activity (De la Sota *et al.*, 1996).

The proliferative response of granulosa cells recovered on day 7 of the oestrous cycle to the stimuli tested in this study was lower than that of cells recovered on day 3. This observation, along with reduced oestradiol production by granulosa cells after 48 h of culture on day 3 versus day 7, indicates that follicles between 4–8 mm in diameter are the most susceptible to the effects of the dominant follicle and to induction of atresia, although it is unclear whether these effects are mediated via direct or indirect mechanisms. It is possible that granulosa cells from day 3 medium-sized follicles have increased proliferative and steroidogenic responses, following exposure to the post-ovulatory FSH peak on day 1 of the oestrous cycle. This would appear unlikely, however, as the oestradiol production of granulosa cells removed from medium-sized follicles on day 3 of the oestrous cycle was similar to that of cultured cells collected from follicles at random stages of the oestrous cycle (see Chapter 6).

In addition to the low proliferative response of day 7 granulosa cells to FSH, these cells also had a low proliferative response to insulin, therefore suggesting that the increase in follicle number associated with high insulin concentrations (Matamoros *et al.*, 1991; Downing and Scaramuzzi, 1991; see Chapter 4) may be due to recruitment rather than to follicle rescue from atresia (Hirshfield, 1989).

Furthermore, the differences in granulosa cell proliferation between medium-sized follicles from day 3 and day 7 of the oestrous cycle in response to FSH extend earlier *in vivo* studies. Adams *et al.* (1993) showed that subordinate follicles respond to exogenous FSH before day 5 of the oestrous cycle, but after day 5, FSH could not rescue subordinate follicles. Similarly, when Ko *et al.* (1991) ablated the dominant follicle by cauterisation on day 3 of the oestrous cycle, the subordinate follicle developed further. In contrast, subordinate follicles did not respond to FSH when the dominant follicle was cauterised on day 5 of the oestrous cycle. Furthermore, it has been observed that superovulatory regimes in animals bearing a dominant follicle showed reduced superovulatory response compared to animals without a dominant follicle (Guibault *et al.*, 1991; Adams *et al.*, 1993). Thus, results described in this Chapter, together with those obtained *in vivo* in response to exogenous FSH, indicate that the proliferative capacity of granulosa cells removed on day 7 in response to FSH is reduced, compared to granulosa cells removed from day 3. Since granulosa cells have limited capacity to replicate (Hirshfield, 1991), cells from day 7 follicles may have undergone more mitotic divisions, and hence, their capacity to replicate further may be reduced.

IGF-I is a putative mediator of gonadotrophin action (Adashi and Rohan, 1992). Cells from large follicles removed on day 3 had higher ($p < 0.01$) proliferative responses to IGF-I than cells from follicles removed on day 7. Since circulating FSH concentrations decline around days 4-5 of the oestrous cycle (*i.e.* the time of dominant follicle selection: Ko *et al.*, 1991; Adams *et al.*, 1993), a greater response of the granulosa cells to IGF-I in newly selected follicles (large follicles, day 3) would give these follicles an advantage when peripheral FSH concentrations fall. Indeed, we have found that IGF-I, even in the absence of FSH, is a potent stimulator

of both steroidogenesis and proliferation of bovine granulosa cells *in vitro* (see Chapter 6).

On a per-cell basis, aromatase activity of granulosa cells after 48 h of culture is decreased by the presence of a dominant follicle, whereas after long-term culture (144 h), cells appear to escape from the inhibitory effect of the dominant follicle. For example, oestradiol production *in vitro* was no longer affected by the hormonal milieu *in vivo*, and cells could respond to the stimulatory hormones tested. As far as we are aware this is the first study that has evaluated the effects of the follicular wave on oestradiol production of granulosa cells in long-term culture. Oestradiol production by granulosa cells at 144 h of culture was similar ($p > 0.05$) for all 3 size categories (Figures 8.1 and 8.4). Furthermore, regardless of the day of the oestrous cycle, granulosa cell oestradiol production remained responsive to FSH, insulin and IGF-I (Figures 8.2 and 8.4). In addition, it is possible that when cells are in culture there is selection against granulosa cells with reduced aromatase activity and at the end of culture only healthy, steroidogenic responsive cells remain. This may explain why oestradiol production per cell did not vary at 144 h of culture between day 3 or day 7.

The results of this study confirm findings described in Chapter 6 that insulin stimulates bovine granulosa cell oestradiol production in a dose-responsive manner, but only up to a dose of 50 ng ml^{-1} . Also, FSH in the presence of physiological concentrations of insulin (0.5 and 5 ng ml^{-1}) stimulates oestradiol production (sheep, Campbell *et al.*, 1996a; cattle, see Chapter 6). However, this stimulatory effect was lost both in response to higher concentrations of FSH ($> 50 \text{ ng ml}^{-1}$) and insulin ($> 50 \text{ ng ml}^{-1}$; Berndtson *et al.*, 1995; see Chapter 6). Furthermore, the oestradiol response of granulosa cells to physiological concentrations of insulin and FSH could not be stimulated further following addition of LR3-IGF-I (Figure 8.4). These results highlight the importance of the synergy of metabolic hormones (insulin and IGF-I) and FSH at physiological concentrations for the induction of granulosa cell proliferation and differentiation (Campbell *et al.*, 1996a; see Chapter 6).

In conclusion, the presence of a dominant follicle *in vivo* inhibited the steroidogenic capacity of granulosa cells from subordinate follicles in short-term culture. However, after long-term culture, the inhibitory effects of the dominant follicle on *in vitro* oestradiol production and on responses to FSH, insulin and IGF-I, were lost. Hence, long-term culture provides a homogeneous cell population to study aromatase activity and differentiation of bovine granulosa cells *in vitro*.

Finally the low proliferative capacity of granulosa cells from day 7 of the oestrous cycle suggest that the increase in follicle number associated with enhanced dietary intake (see Chapter 4) and in response to gonadotrophin treatment is due to increased recruitment of follicles, rather than rescuing follicles from atresia.

9. FOLLICLE GROWTH AND ANTRUM FORMATION OF BOVINE PREANTRAL FOLLICLES IN LONG-TERM CULTURE *IN VITRO*.

9.1 ABSTRACT

Culture of preantral follicles has important biotechnological implications through its potential to produce large quantities of oocytes for embryo transfer. The success of follicle culture in domestic species has been hindered by follicles losing their three-dimensional architecture and granulosa cells plating down, often leaving a denuded oocyte. A long-term culture system for bovine preantral follicles that overcomes these problems is described. Bovine preantral follicles ($166 \pm 2.15 \mu\text{m}$), surrounded by theca cells, were isolated from ovarian cortical slices. Follicles were cultured under conditions known to maintain granulosa cell viability *in vitro*. The effects of EGF, IGF-I, FSH and co-culture with bovine granulosa cells on preantral follicle growth were analysed. Follicle and oocyte diameter increased with time in culture. FSH, IGF-I and EGF stimulated ($p < 0.05$) follicle growth rate, but did not stimulate oocyte growth. Co-culture with granulosa cells inhibited FSH/IGF-I stimulated growth. Most follicles maintained their morphology throughout culture with the presence of a theca layer and basement membrane surrounding the granulosa cells. Antrum formation (confirmed by confocal microscopy) occurred between days 10 to 28 of culture. The probability of follicles reaching antrum development was 0.19 for control follicles. The addition of growth factors or FSH increased ($p < 0.05$) the probability of developing an antrum to 0.55. Follicular growth appeared to be halted by slower growth of the basement membrane, as occasionally growing follicles burst the basement membrane, extruding the granulosa cells. In conclusion, a preantral follicle culture system in which follicle morphology can be maintained for up to 28 days has been developed. In this system FSH, EGF and IGF-I stimulated follicle growth and enhanced antrum formation. This culture system may provide a valuable

technique for studying the regulation of early follicular development, and for production of oocytes for nuclear/embryo transfer.

9.2 INTRODUCTION

Recent advances in animal cloning techniques (Campbell *et al.*, 1996b; Wilmut *et al.*, 1997) have opened up broader horizons in biotechnology. However, these techniques depend on the production of fully developed oocytes. At the moment, their availability is limited to the number of antral follicles present in the ovaries. The development of a preantral follicle culture system that can potentially produce large quantities of oocytes of uniform developmental status will advance these techniques further. Additionally, it may make possible the preservation and long-term storage of the female genoplasm.

Early follicular development is a lengthy process (Lussier *et al.*, 1987; Gougeon, 1996) and its regulation remains largely unknown. Nonetheless, it is clear that successful culture of murine oocytes, fertilisation and further developmental competence are only achieved if the three-dimensional organisation of the granulosa cells around the oocyte is maintained throughout culture (Eppig and O'Brien, 1996). Indeed, the oocyte is dependent on the surrounding granulosa cells from which it receives its nutrients (Heller *et al.*, 1981). Nutrient transport between the granulosa cells and the oocyte is achieved by extensive gap junction communication (Heralds and Schultz, 1984; Buccione *et al.*, 1990). In addition, granulosa cells are thought to produce a "maturation inhibiting factor" that maintains the oocyte in meiotic arrest. Removal of the granulosa cells (Eppig and Downs, 1984), or disruption of the gap junction communications between granulosa cells and the oocyte results in spontaneous germinal vesicle breakdown (Eppig and Downs, 1987).

The success of follicle culture in domestic species has been hindered by a loss of three-dimensional architecture, and plating down of granulosa cells, often leaving a denuded oocyte (Eppig and Schroeder, 1989). In Chapter 6 a long-term granulosa cell culture system is described that allows non-luteinised cells to remain responsive to physiological concentrations of FSH, insulin and IGF-I. Furthermore, granulosa

cells cultured in this system form cell aggregates with extensive gap junction communications between cells (see Chapter 7). In this Chapter, the granulosa cell culture system is adapted for bovine preantral follicles. This culture system can sustain long-term culture of preantral follicles. Follicles maintained their cellular organisation and general architecture, and follicle growth and enhanced antrum formation can be stimulated by FSH, EGF and IGF-I. This culture system may provide a valuable approach for study of the regulation of early follicular development.

9.3 MATERIALS AND METHODS

9.3.1 *PREANTRAL FOLLICLE ISOLATION AND CULTURE*

On five occasions, between 16 to 28 healthy bovine preantral follicles were dissected out, as described in Chapter 2, and cultured individually in 96 well plates with 250 μ l of culture medium supplemented with 10 ng/ml of insulin. Follicle treatments included the growth factors, EGF (0.5 ng/ml; Toyoba Co. Ltd. Osaka Japan), and long-R3 IGF-1 (10 ng/ml), and FSH (1 ng/ml) alone or in combination with the growth factors, and IGF-I/FSH co-cultured with granulosa cells obtained from antral follicles (2-4 mm in diameter).

Cultures were originally intended to last for 6 days. However, after the first two cultures 100% of the follicles had maintained a normal and healthy appearance. Therefore, further cultures lasted for 28 days (cultures 3 and 4) or 19 days (culture 5).

9.3.2 *STATISTICAL ANALYSES*

The effects of treatment and day of culture on follicular and oocyte diameter were analysed by regression analysis testing for homogeneity of regression among treatments and allowing for the variation between cultures. The effects of the initial diameter of the oocyte or follicle on their growth in culture were tested by including them as covariables in the analysis. The effects of treatment on antrum development or follicle degeneration were analysed by generalised linear mixed models, allowing

for the random effect of experiment (Breslow and Clayton, 1988). The cumulative effect of treatment versus control was assessed using a Bonferroni's adjustment to decrease the probability of significance (Maxwell and Delaney, 1990). Follicle degeneration was considered to have occurred in those follicles that deteriorated without forming an antral cavity. The effects of treatment on the day of antrum formation or degeneration of follicles that did not form an antrum was tested by survival analysis. Viable non-antral follicles remaining at the end of each culture (4/27, 3/24 and 3/18 for culture 3, 4 and 5 respectively) were considered as right-censored observations for analysis. The effects of the oocyte, or follicle diameter before culture, on the day of antrum formation or degeneration were also tested.

9.4 RESULTS

9.4.1 EFFECTS OF TREATMENT ON FOLLICLE GROWTH

Mean preantral follicle diameter was $166 \pm 2.15 \mu\text{m}$ at the beginning of the cultures. Regression analysis showed an increase in follicle diameter with day in culture (Figure 9.1; Table 9.1), with a significant ($P < 0.01$) quadratic effect of the day of culture over time ($R^2 = 0.74$). This indicated a rapid increase in follicle diameter during the first week of culture that then declined after 8-10 days of culture. Treatment with FSH, IGF-I, FSH/IGF-I and EGF enhanced ($P < 0.05$) the growth rate of preantral follicles. In contrast, the presence of granulosa cells inhibited ($P > 0.05$) FSH/IGF-I stimulation (Figure 9.1). The diameter of the follicle at the beginning of the culture was significantly (0.84 ; $p < 0.05$) below one, indicating that the larger a follicle is at the beginning of the culture, the lower its growth in culture. Oocyte diameter however, had no effect ($P > 0.05$) on the growth of follicles in culture.

As observed for follicle diameter, oocyte diameter increased with time in culture (regression $y = 57.12 + 0.62 \text{ day}$; $R^2 = 0.47$). However, there was no effect of treatment ($P > 0.10$) on oocyte growth.

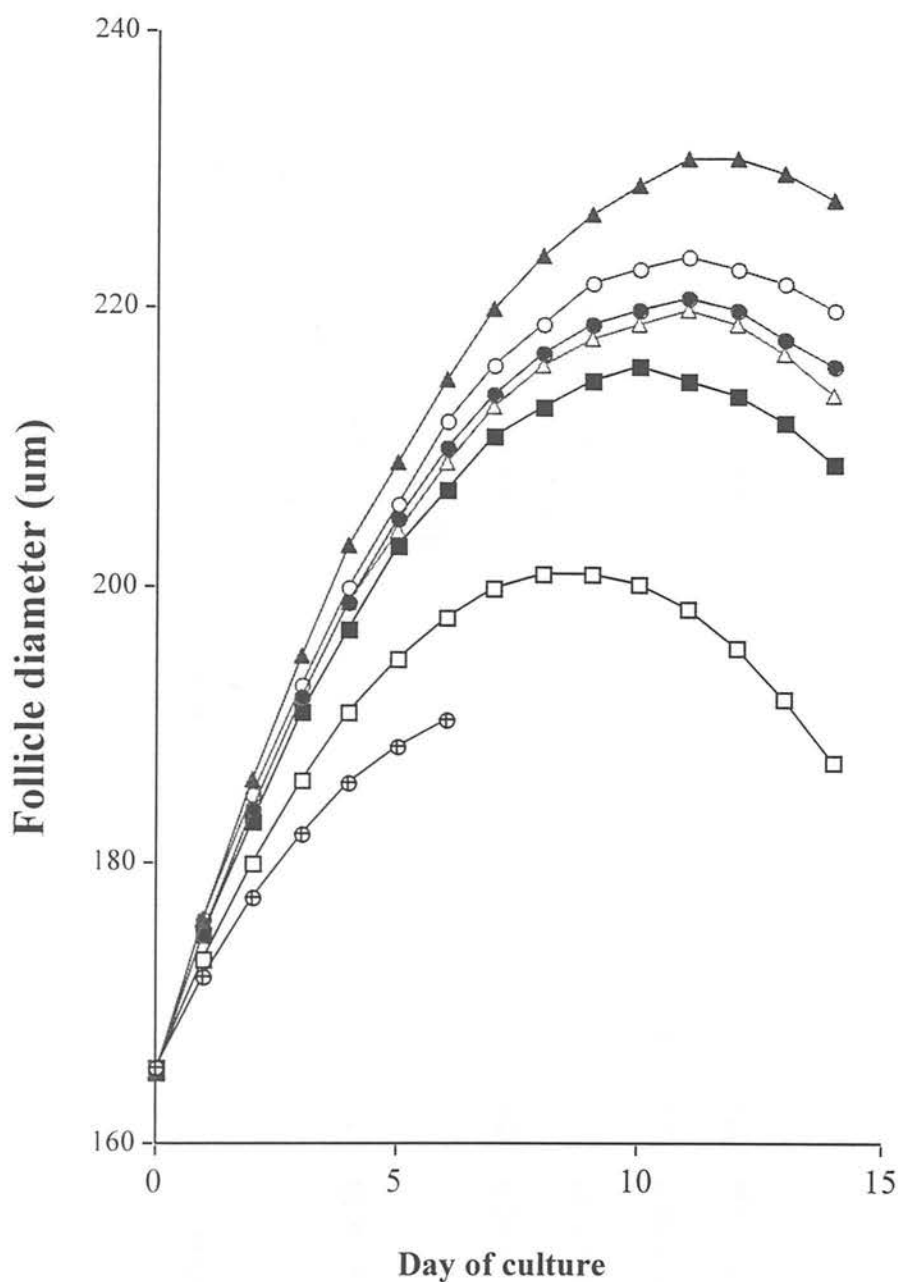


Figure 9.1. Regression curves for the growth of preantral follicles cultured in granulosa-cell culture medium either without hormone treatment (Control; □), or with EGF (0.5 ng/ml; ▲), FSH (1 ng/ml; ●), FSH/EGF (■), IGF (1 ng/ml; △), IGF/FSH (○), and IGF/FSH in co-culture with granulosa cells (⊕). $R^2 = 0.74$.

Table 9.1. Diameter of preantral follicle cultured in granulosa-cell culture medium either without hormone treatment (Control), or with FSH (1 ng/ml), EGF (0.5 ng/ml), FSH/EGF, IGF (1 ng/ml), IGF/FSH, and IGF/FSH in co-culture with granulosa cells. Values are least square means \pm SEM.

Days of culture	control	FSH	EGF	FSH/EGF	IGF	IGF/FSH	GC
0	163.4 \pm 4.68	166 \pm 4.58	150 \pm 10.1	153.8 \pm 10.16	167.8 \pm 5.185	172.7 \pm 3.9	179.99 \pm 8.2
2						188.9 \pm 6.2	196.52 \pm 8.2
4	180.9 \pm 10.9	184 \pm 10.9	195 \pm 11.1	199.3 \pm 10.16		203.4 \pm 6.4	198.05 \pm 8.2
6	197.6 \pm 5.35	219 \pm 5.22			213 \pm 5.356	213.8 \pm 4.2	207.46 \pm 8.65
10	207.3 \pm 10.9	208 \pm 10.9	211 \pm 10.1	211.6 \pm 10.16			
14	183.6 \pm 5.18	217 \pm 5.04	216 \pm 10.1	200.3 \pm 10.16	217.2 \pm 6.32	230.7 \pm 6.3	

9.4.2 FOLLICLE MORPHOLOGY

Immediately after isolation, preantral follicle granulosa cells were surrounding a spherical oocyte. The granulosa cells were limited by an intact basement membrane and an outer thecal layer (Figure 9.2; day 0). This basic morphology of the follicle was preserved throughout the culture period. Follicles remained in an oval or spherical shape and did not attach to the well surface. As follicle diameter increased with time in culture, there was an increase in granulosa cell number, and an increase in the volume of the follicle (Figure 9.2; day 0). After an extended period of culture (range 10 to 28) antral spaces appeared within some follicles. These antral spaces grew in size to form antral cavities (Figure 9.2-9.5).

9.4.3 ANTRUM FORMATION AND/OR FOLLICLE DEGENERATION

The mean probability of antrum formation or degeneration is shown by treatment in Table 9.2. There were no differences ($P>0.05$) in the mean probability of antrum formation among treatments. However, the cumulative mean probability of antrum formation for treated follicles was higher ($P<0.05$) than for control follicles. The estimated probability for follicle degeneration by treatment did not differ ($P>0.05$), nor did the cumulative probability of treated follicles versus control follicles ($P>0.05$).

Survival analysis showed that the time from the beginning of culture to antrum development was not affected by the treatment applied ($P>0.05$), nor by oocyte or follicle diameter when isolated. Similarly, treatment did not affect ($P>0.05$) day of follicle degeneration. However, oocyte diameter at beginning of culture had a negative relationship (regression coefficient = -0.08; $p<0.05$) with day of follicle degeneration.

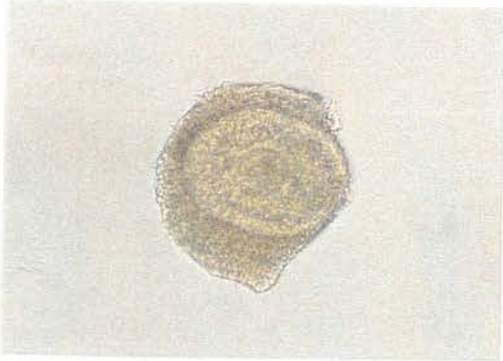
A common characteristic of follicle degeneration was extrusion of the granulosa cells from the follicle (Figure 9.2b; 9.3e,f) after bursting the basement membrane. This was observed for a range of follicles, whether or not they had previously formed an antral cavity. These granulosa cells extruded from within the follicular walls remained in close proximity, forming clumps that remained viable.

Figure 9.2. Photomicrographs of bovine preantral follicles after isolation (day 0) and after different times in culture. Notice that follicles remained spherical throughout culture and retained their three-dimensional architecture. Follicle "a" was cultured for 12 days and an antrum was visible at day 10 (day 10) of culture. Follicle "b" was in cultured for 25 days and although it doubled in size, it did not form an antral cavity and degenerated by rupture of the basement membrane and extrusion of granulosa cells.

a. Day 0



b. Day 0



a. Day 4



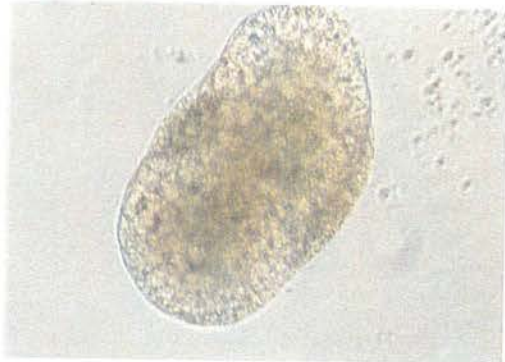
b. Day 19



a. Day 10



b. Day 24



a. Day 12



b. Day 25

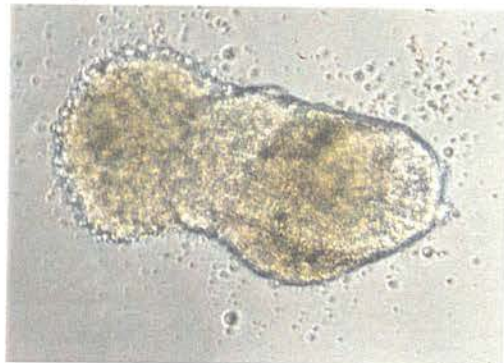


Figure 9.3. Photomicrographs of bovine preantral follicles that developed antra after 8 (a), 10 (b), 14 (c) and 14 (d) days or degenerated by rupture of the basement membrane after 25 days in culture (e, f).

a.



b.



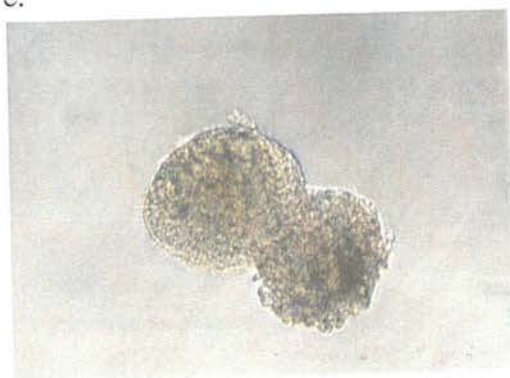
c.



d.



e.



f.

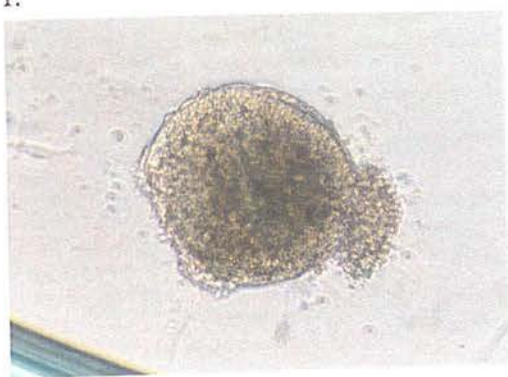


Figure 9.4. Confocal photomicrographs of preantral follicles, demonstrating the presence of an antrum within the granulosa cells. Scale bar = 100 μm .

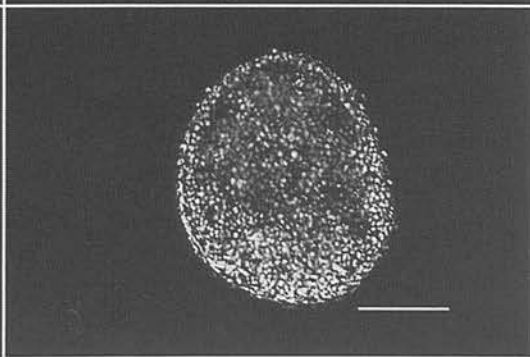
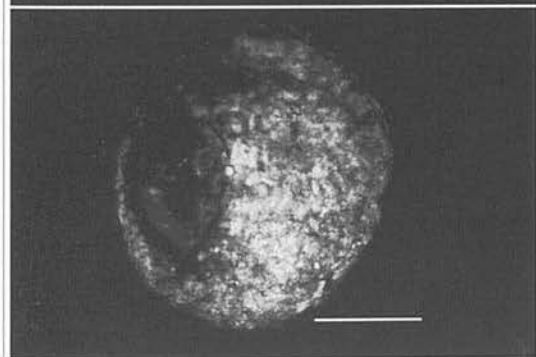
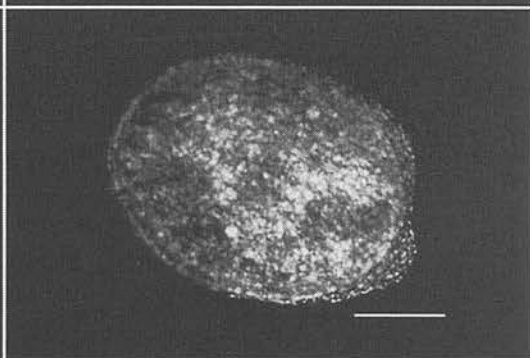
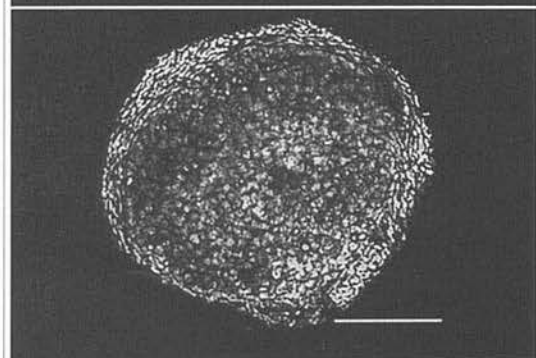
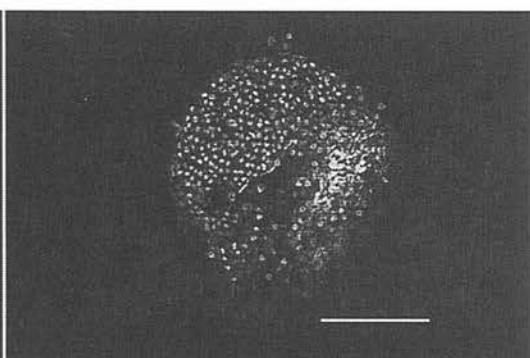
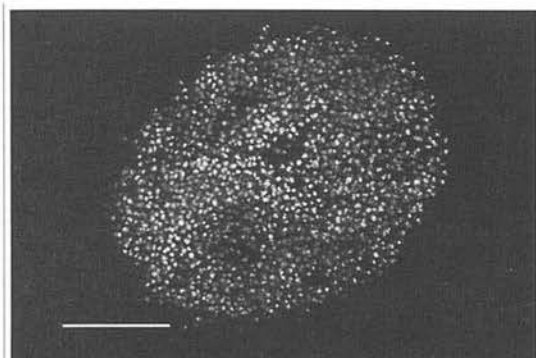


Figure 9.5. Confocal Z-serial sections of a preantral follicle after 25 days in culture and the development of an antral cavity. 10 μm sections were taken from the edge of the theca cell layer towards the centre of the follicle. Scale bar = 50 μm .

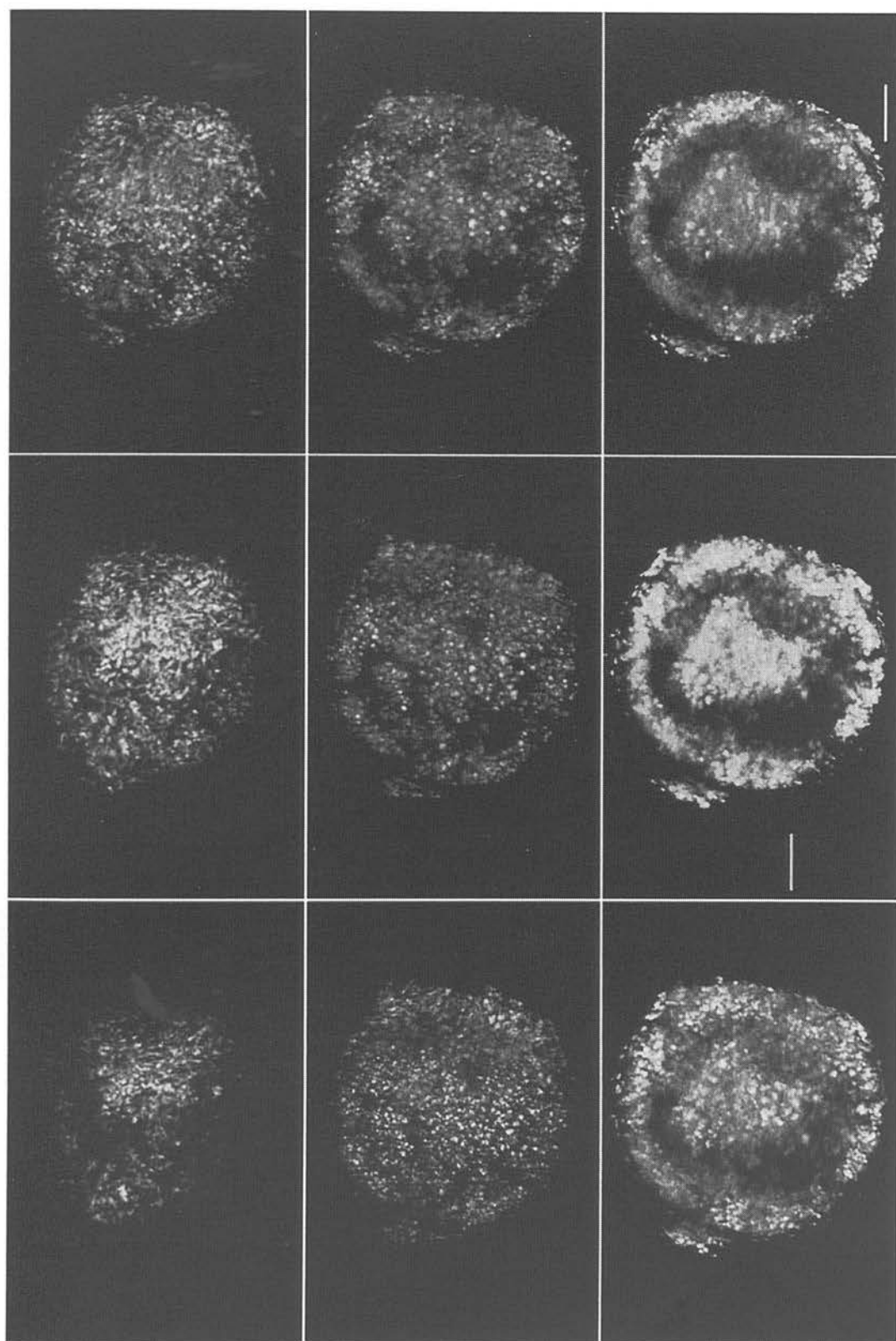


Table 9.2. Calculated mean probability values for antrum formation or degeneration of untreated follicles (control) or treated follicles. Follicles were treated with either growth factors (IGF-I or EGF), FSH or a combination of FSH and growth factors. The pooled probability for antrum formation for the treated follicles is shown. a, b indicate significant difference ($p<0.05$). ne=non-estimated.

Developmental Status	Control	FSH	IGF-I	FSH/IGF-I	EGF	FSH/EGF	Cumulative treatment probability
Antrum	0.20 ^a	0.68 ^a	0.54 ^a	0.59 ^a	0.36 ^a	0.36 ^a	0.55 ^b
Degeneration	0.53 ^a	0.33 ^a	0.16 ^a	0.47 ^a	0.51 ^a	ne	0.30 ^a

9.5 DISCUSSION

The culture of bovine preantral follicles was successfully achieved. Follicle growth and architecture were maintained *in vitro* for up to 28 days. Additionally, the effects of FSH, EGF and IGF-I on growth and antrum formation in preantral follicles were demonstrated.

As far as we are aware, this is the first report of development of antrum formation in cultured bovine preantral follicles. Follicular antrum development has been accomplished in murine preantral follicles (Spears *et al.*, 1994; Boland *et al.*, 1993), but has been unsuccessful in larger domestic species. The reason for this difference may be due to important species differences. First, the time needed to develop an antral follicle varies considerably between species. Whilst ovulatory size is reached in the mouse when the follicle is 0.5 mm in diameter (Telfer, 1996), in cattle the ovulatory size is >1.6 cm. Furthermore, the length of time taken for cultured murine preantral follicles to grow to Graafian follicles was 6 days (Boland *et al.*, 1993), and corresponds to the length of time taken by follicles *in vivo*. In contrast, follicular development from the preantral stages to ovulatory size in cattle and sheep can take a number of months (Lussier *et al.*, 1987; Gosden *et al.*, 1994). Therefore, in order for a follicle to develop to the preovulatory stage, much longer periods of culture may be needed.

Long-term culture requirements have hindered the advance of preantral follicle cultures. To date, no culture system is reported that can maintain follicular architecture for longer than 12 days (Telfer, 1996). Thus the adaptation of the granulosa cell culture system that maintains phenotypically non-luteinised granulosa cells (see Chapter 6) in long-term culture has enabled the culture of bovine preantral follicles, allowing studies of follicle growth and maturation (antrum formation) whilst keeping follicular architecture intact.

Preantral follicles grew in culture, even in the absence of trophic hormones. This growth was probably promoted by the presence of insulin in the culture media. We have shown that insulin promotes the proliferation of granulosa cells under similar

cultural conditions (Campbell *et al.*, 1996a; see Chapter 6). Follicular growth may also be regulated by substances produced within the follicle. Indeed, numerous putative autocrine/paracrine follicle mediators have been investigated. Activin is a granulosa cell product which increased thymidine uptake by mouse preantral follicle granulosa cells (Li *et al.*, 1995). Similarly, granulosa cell produced-inhibin can stimulate oestradiol and androstenedione production by granulosa and theca cells respectively (Campbell *et al.*, 1995). Granulosa cells of preantral follicles produce a theca differentiation factor (Magarelli *et al.*, 1996) that stimulates androstenedione production by theca cells. We have found IGF-I mRNA in thecal cells and IGF-II mRNA expression in both theca and granulosa cells of bovine antral (3-8 mm) follicles (see Chapter 10). Indeed, Armstrong *et al.* (1997) found IGF-II mRNA expression in the theca layer of bovine follicles from 1-2 mm in diameter. Thecal cells also produce EGF/TGF α (Ojeda and Dissen, 1994) and these were shown to stimulate proliferation of theca and granulosa cells in the mouse and sheep (Ojeda and Dissen, 1994; Campbell *et al.*, 1996a).

Follicular growth may also be influenced by the interference from an inhibitory factor within the ovary. Compelling evidence for a local inhibitory factor(s) within the ovary is the activation of primordial follicles in ovarian tissue slices (Wandji *et al.*, 1996; Braw-Tal and Yossefi, 1997). This inhibitory factor(s) acts on follicles of different size ranges. When murine preantral follicles were cultured in groups (Nayadu and Osborn, 1992), or in contact with each other (Nayadu and Osborn, 1992; Spears *et al.*, 1996), the growth of some follicles was inhibited. In the current study, the addition of FSH, EGF and/or IGF-I stimulated higher rates of follicular growth compared to control follicles, confirming the stimulatory effects of FSH and growth factors. In contrast, co-culture of preantral follicles with granulosa cells from antral (2-4 mm) follicles inhibited the stimulatory actions of FSH/IGF-I on follicle growth. However, although smaller than their counterparts, these follicles otherwise appeared healthy. Whether attenuation of follicular growth was due to a direct effect of a putative inhibitory factor secreted by more mature granulosa cells, or due simply to competition for nutrients and stimulatory hormones could not be determined.

The probability of antrum formation in follicles treated with FSH, IGF-I or EGF was over 0.50 and was associated with a significant increase in follicular diameter. Antrum formation may regulate acid-base balance and respiratory gases (Gosden *et al.*, 1988) once the follicle becomes too large for nutrients and oxygen to reach the cells by passive diffusion (around 200 μm in diameter). Follicle development stopped in murine follicles, grown *in vitro*, at the preantral stage in the absence of FSH (Boland *et al.*, 1993). No differences in follicle growth or in the probability of antrum formation were observed in this study, with growth factor treatments that did or did not contain FSH. Although we cannot discount the effects of gonadotrophic priming of the follicles previous to their isolation, these results may indicate that the threshold of gonadotrophin dependence varies between species, depending on the size of the preovulatory follicle. Accordingly in the absence of FSH, preantral follicle growth stops at the preantral stage in hypophysectomised mice (Wang and Greenwald, 1993a), at 2.5 mm in diameter in hypophysectomised and gonadotrophin depleted sheep (Dufour *et al.*, 1979; Picton *et al.*, 1990) and around 3-4 mm in gonadotrophin suppressed cattle (Gong *et al.*, 1996a). The use of this culture system will enable study of the regulation of early follicular growth and antrum formation in preantral follicles.

In addition to growth of follicles, oocytes within these follicles grew with time in culture. As found by others (Braw-Tal and Yossefi, 1997), FSH and growth factors appear to have no effects on oocyte development. However, there was continuous growth of the oocyte within the follicle, indicating that follicles were physiologically healthy and that intercellular communications between the granulosa cells and the oocyte were maintained during the period of culture. The oocyte depends on granulosa cells for its nourishment (Buccione *et al.*, 1990; Eppig, 1991), and if these communications are disrupted, the oocyte degenerates. Oocyte growth and maturation occurs even in the absence of theca cells, in so-called granulosa-oocyte complexes (GOC). In GOCs, oocytes mature and can be fertilised and develop into an embryo (Spears *et al.*, 1994; Eppig and Schroeder, 1989; Eppig and O'Brien, 1996), highlighting the importance of the granulosa cell in the nourishment of the oocyte. Here again, species differences may dictate specific culture requirements. In

the mouse, the preantral follicle has to grow for a further 4 days to attain preovulatory size (Telfer, 1996), and the oocyte is almost fully developed in the preantral follicle. In sheep, Gosden *et al.* (1994) observed that it took 3-4 months for animals to regain cyclicity after ovariectomy and autotransplantation with ovarian grafts. In the bovine preantral follicle, it is estimated that a further 40 days are needed for the follicle to reach the preovulatory size, and the oocyte has to double its size during this time (Telfer, 1996). Therefore, oocyte maturity in the murine preantral follicle may permit the culture of GOC for a short period, yielding viable oocytes. In this study, follicles in which the GOC were extruded after bursting the follicular wall (Figure 9.2b; 9.3e,f) maintained the three dimensional architecture of the granulosa cells surrounding the oocyte. Thus, further studies are needed to evaluate whether these GOC are able to survive for long-term periods in culture and to evaluate oocyte growth and competence.

Follicular growth is accompanied by extensive tissue remodelling of the theca interna and basement membrane surrounding the granulosa cells. In this study, the 30% increase in follicle diameter corresponded to a 72% increase in follicle surface area. Following culture of preantral follicles, thecal tissue was thinner and appeared to be stretched (Figure 9.2) compared to follicles at the time of follicle collection (Figure 9.2; day 0). Furthermore, some follicles grew by rupturing the basement membrane and theca layer, thereby extruding the GOC. Basement membrane remodelling involves the selective degradation and synthesis of extracellular matrix (ECM) proteins. ECM degradation involves complex interactions between proteolytic enzymes (matrix metalloproteinases) and their regulators (Luck, 1994). The secretion of gelatinase activity has been demonstrated in theca cells in serum-free culture (Smith *et al.*, 1997). In addition, both granulosa and theca cells produce extracellular matrix components (Luck, 1994). However, whether follicular cells *in vitro* are able to produce the enzymes and substrates needed to assemble the basement membrane at a rate of growth required to maintain the integrity of the basement membrane of growing follicles for long-term periods in culture needs further investigation.

In conclusion, sustained growth of bovine preantral follicles and their oocytes for long-term culture periods has been achieved. Under these culture conditions, follicles maintained the cellular organisation required for the appropriate nourishment of the oocytes within the follicle, and were responsive to stimulatory effects of FSH, EGF, IGF-I and possibly insulin. For the first time, the growth of bovine preantral follicles up to the antral stage is described. This culture system should provide a valuable model for the study of the regulation of early follicular growth and antral development.

10. INSULIN-LIKE GROWTH FACTOR (IGF)-I PRODUCTION AND EXPRESSION OF IGF-I AND -II BY BOVINE GRANULOSA AND THECA CELLS *IN VIVO* AND *IN VITRO*.

10.1 ABSTRACT

IGFs are potent mediators of steroidogenesis and proliferation of granulosa cells and can act through autocrine and/or paracrine mechanisms. However, the detailed pattern of mRNA expression and production of IGFs in bovine follicles has not been determined. The objectives of this study were to: i) determine whether bovine granulosa cells produce IGF-I *in vitro*, ii) evaluate the pattern of IGF-I and II gene expression in bovine granulosa and theca cells *in vivo* and *in vitro*, and iii) determine the effects of gonadotrophin treatment on IGF-I and II gene expression.

In the first experiment, granulosa cells from medium-sized follicles (4-8 mm) were cultured in serum-free culture media supplemented with insulin (0-5000 ng/ml) and FSH (0-10 ng/ml). Culture media were replaced every 48 h and cultures were terminated after 144 hours. Cellular IGF-I production was measured using a radioimmunoassay for nonextracted culture samples, and the effects of insulin and FSH on IGF-I production were tested. IGF-I levels were below the detection limit, and neither insulin nor FSH induced detectable levels of IGF-I production.

In the second experiment, granulosa and thecal cells were isolated and cultured for 96 h. Granulosa cells were cultured with FSH (0, 1 or 50 ng/ml) or LH (0, 100 ng/ml) and theca cells were cultured with LH (0 or 100 ng/ml). Total RNA was extracted either prior to or after 96 h of culture, and messenger RNAs for IGF-I and IGF-II were detected by reverse transcriptase PCR using oligomers homologous with sequences within the bovine IGF-I and IGF-II cDNAs. IGF-I mRNA was found in thecal cells, but not in granulosa either before or after culture. In contrast, IGF-II mRNA was expressed in both theca and granulosa cells. Thus IGF-I is produced by

theca cells, but not by granulosa cells, whereas IGF-II is produced by both theca and granulosa cells. These results support a paracrine action of IGF-I on granulosa cells, and a possible autocrine and/or paracrine action of IGF-II on granulosa cells.

10.2 INTRODUCTION

Insulin-like growth factors (IGFs) are putative modulators of follicular growth and development, and regulate the actions of gonadotrophins in the ovary (Guidice, 1992; Adashi, 1995; Hammond *et al.*, 1993). IGF-I stimulates steroidogenesis and cell proliferation in granulosa cells of rat (Adashi, 1992b), pig (Howard and Ford, 1994), sheep (Campbell *et al.*, 1996a), and cattle (Armstrong *et al.*, 1996a; see Chapter 6). The thecal layer is also a site of IGF-I action in a number of species (porcine: Caubo *et al.*, 1989; ovine: Campbell *et al.*, 1996a; bovine: Stewart *et al.*, 1995; Spicer and Stewart, 1996a). Although IGF-II has not been studied as extensively as IGF-I, its effects on proliferation and steroidogenesis have also been demonstrated (Campbell *et al.*, 1995; Hammond *et al.*, 1991; Monget and Monniaux, 1995).

IGF-I and -II have been measured in bovine follicular fluid, and changes in concentrations have been described which vary according to the stage of follicular development (Echternkamp *et al.*, 1990; Monget *et al.*, 1993). In addition, bovine granulosa cells *in vitro* produce immunoreactive IGF-I (Spicer *et al.*, 1993b; see Chapter 3). Furthermore, IGF-I and -II mRNA expression in the ovary is tissue- and species-specific, and is developmentally regulated. For example, in the human ovary, IGF-I mRNA is localised in the theca layer of small, but not dominant follicles (El-Roeiy *et al.*, 1993), whereas IGF-II mRNA is exclusively localised in the granulosa cells (Zhou and Bondy, 1993; El-Roeiy *et al.*, 1993). In contrast, in the rat, IGF-I mRNA is localised in the granulosa cells (Hernandez, 1995; Botero *et al.*, 1993) and IGF-II in the theca layer (Hernandez, 1995). Nonetheless, in ruminants it is unclear which cells within the follicle (if any) are responsible for the secretion of IGF-I and -II. Two studies investigating *in situ* localisation of mRNA IGF-I in the sheep ovary have given contradictory results. Whilst one report indicated the expression of

mRNA for IGF-I in both theca and granulosa cells (Leewenberg *et al.*, 1995), another group, using the same approach, reported localisation of mRNA IGF-II confined to the theca, with no IGF-I expression in the follicle (Perks *et al.*, 1995). However, the expression of IGF-I mRNA was demonstrated in granulosa-derived luteal cells (Perks *et al.*, 1995).

In Chapters 3, 6 and 8 the stimulatory effects of IGF-I on bovine granulosa cells was demonstrated in culture. However, no detectable IGF-I was produced by non-luteinised bovine granulosa cells in culture, using an IGF-I radioimmunoassay that overcomes interference by IGFBPs (see Chapter 3). In this study, we characterised IGF-I and -II mRNA expression *in vivo* and *in vitro* and IGF-I production by bovine granulosa and theca cells *in vitro*. The effects of gonadotrophins on IGF-I and -II mRNA expression was also evaluated in physiological relevant theca and granulosa cell culture systems.

10.3 MATERIALS AND METHODS

10.3.1 EXPERIMENT 1.

Granulosa cells were isolated from medium-sized follicles, as described in Chapter 2, and seeded (75×10^3 viable cells per well) in quadruplicate in 96 well plates. The cells were cultured for up to 6 days with 1 ng ml^{-1} of FSH, and the effect of dose of insulin ($0\text{-}500 \text{ ng ml}^{-1}$) tested. Alternatively, granulosa cells were cultured with 10 ng ml^{-1} of insulin and the effects of FSH ($0\text{-}10 \text{ ng ml}^{-1}$) on oestradiol and IGF-I production tested. The experiment was repeated 3 times.

10.3.2 EXPERIMENT 2.

Granulosa and theca cells from medium-sized follicles were isolated and pooled. Three million cells were cultured in duplicate in 10 ml of the appropriate culture medium for up to 96 h. Granulosa cell cultures were supplemented with 10 ng ml^{-1} of insulin and with 0, 1, or 50 ng ml^{-1} FSH or 0 or 100 ng ml^{-1} LH. Theca cell cultures were supplemented with insulin (100 ng ml^{-1}) and 0 or 100 ng ml^{-1} LH.

Total RNA was extracted from both granulosa and theca cells before or after 96 h of culture. The expression of mRNAs for IGF-I and -II in theca and granulosa cells before or after culture was analysed by RT-PCR using primers complementary to IGF-I (Fotsis *et al.*, 1990) or IGF-II (Brown *et al.*, 1990) sequences (Table 10.1). Liver mRNA was included as a positive control for the expression of both IGF-I and -II. The experiment was repeated 3 times.

Table 10.1. IGF-I and IGF-II primers sequence used in RT-PCR reactions. Amplified products correspond to nucleotides 156-351 and 7-160 of the IGF-I and IGF-II complementary DNA respectively.

	Primer	size (bp)
IGF-I	upstream 5'CCTCTGCGGGGCTGAGTTGGT-3'	195
	downstream 5'CGACTTGGCGGGCTTGAGAGGC-3'	
IGF-II	upstream 5'TCTGTGCGGCGGGGAGCTGGT-3'	153
	downstream 5'AGTCTCCAGCAGGGCCAGGTCG-3'	

10.3.3 RADIOIMMUNOASSAYS

Oestradiol and progesterone were measured as previously described, the inter- and intra-assay coefficients of variation were 12.6% and 7.8% for oestradiol and the intra-assay coefficients of variation for progesterone and androstenedione were 4.8% and 6.7% respectively. IGF-I was measured by the IGF-I competitive RIA as described in Chapter 3. The intra-assay coefficients of variation was 7.7%.

10.3.4 STATISTICAL ANALYSES

Data on steroid production were analysed using the mixed models, containing as independent variables insulin, FSH and LH (fixed effects), and allowing for variation due to culture and different pools of cells (random effects).

10.4 RESULTS

10.4.1 HORMONE PRODUCTION

In the first experiment, oestradiol production by granulosa cells increased ($P<0.01$) in a dose-responsive manner between 0 and 1 ng ml⁻¹ of FSH (Figure 10.1a). Similarly, insulin (Figure 10.1b) stimulated ($P<0.01$) oestradiol production by granulosa cells, reaching maximal production at 50 ng ml⁻¹ of insulin. However, at the dose tested neither FSH nor insulin stimulated granulosa cells to produce detectable amounts of IGF-I.

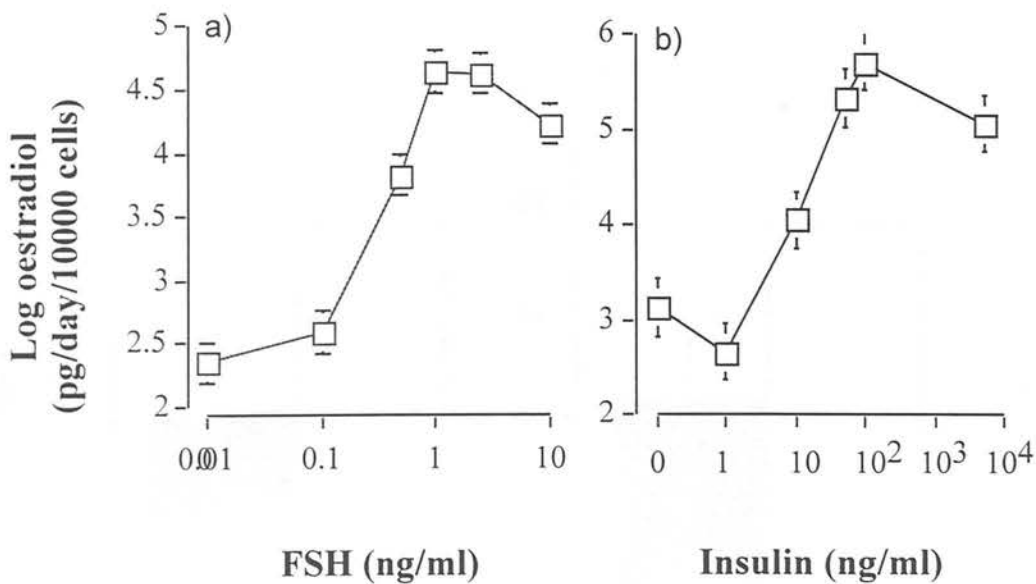


Figure 10.1. Effect of FSH (a) and insulin (b) on oestradiol production per 10,000 bovine granulosa cells from medium-sized (4-8 mm) follicles after 144 h of culture. The effect of FSH was evaluated in cells cultured with 10 ng ml⁻¹ of insulin. The effect of insulin was evaluated with cells cultured with 1 ng ml⁻¹ FSH. Values are least square means ± SEM.

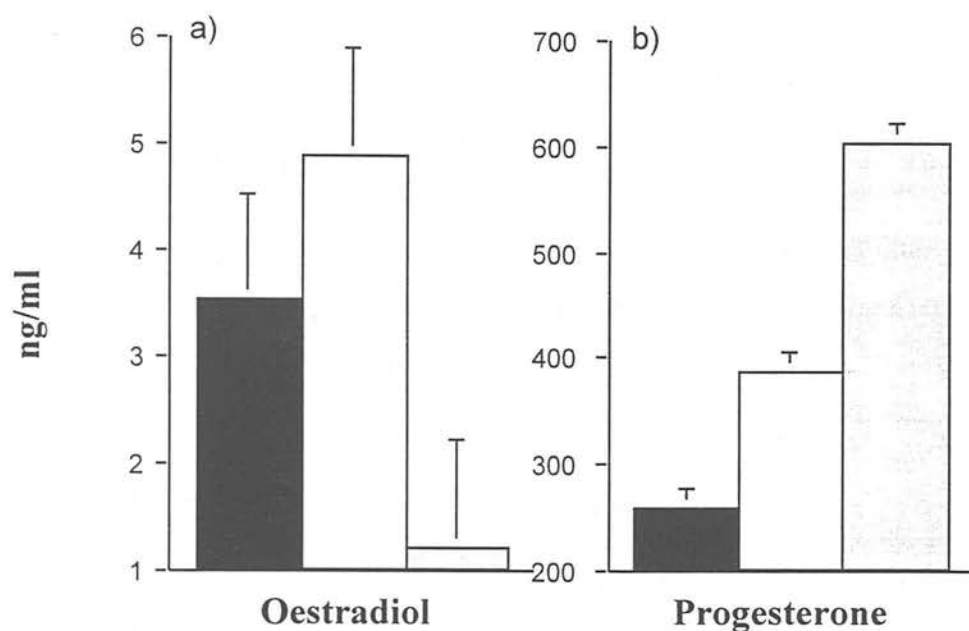


Figure 10.2. Oestradiol (a) and progesterone (b) production by bovine granulosa cells from medium-sized (4-8 mm) follicles after 96 h of culture with 10 ng ml⁻¹ of insulin and 0 (■), 1 (□), or 50 (□) ng ml⁻¹ FSH. Values are least square means \pm SEM.

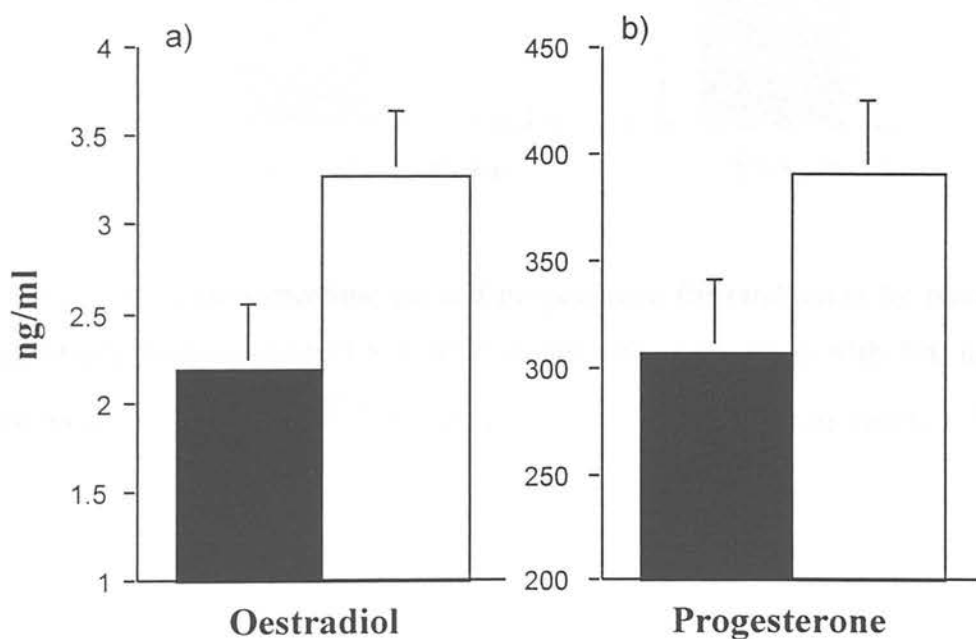


Figure 10.3. Oestradiol (a) and progesterone (b) production by bovine granulosa cells from medium-sized (4-8 mm) follicles culture for 96 h with 10 ng ml⁻¹ of insulin, 1 ng ml⁻¹ FSH and 0 (■) or 100 (□) ng ml⁻¹ LH. Values are least square means \pm SEM.

In the second experiment (Figure 10.2), 50 ng ml⁻¹ of FSH provoked a reduction in granulosa cells (Figure 10.2a) oestradiol production ($P<0.01$), compared to 0 or 1 ng ml⁻¹ of FSH. In contrast, progesterone production increased ($P<0.01$) with increasing FSH dose (Figure 10.2b). LH also increased progesterone production by granulosa cells (Figure 10.3b), but had no effect on oestradiol production (Figure 10.3a). LH stimulated an increase in androstenedione (Figure 10.4a) and progesterone (Figure 10.4b) production by theca cells.

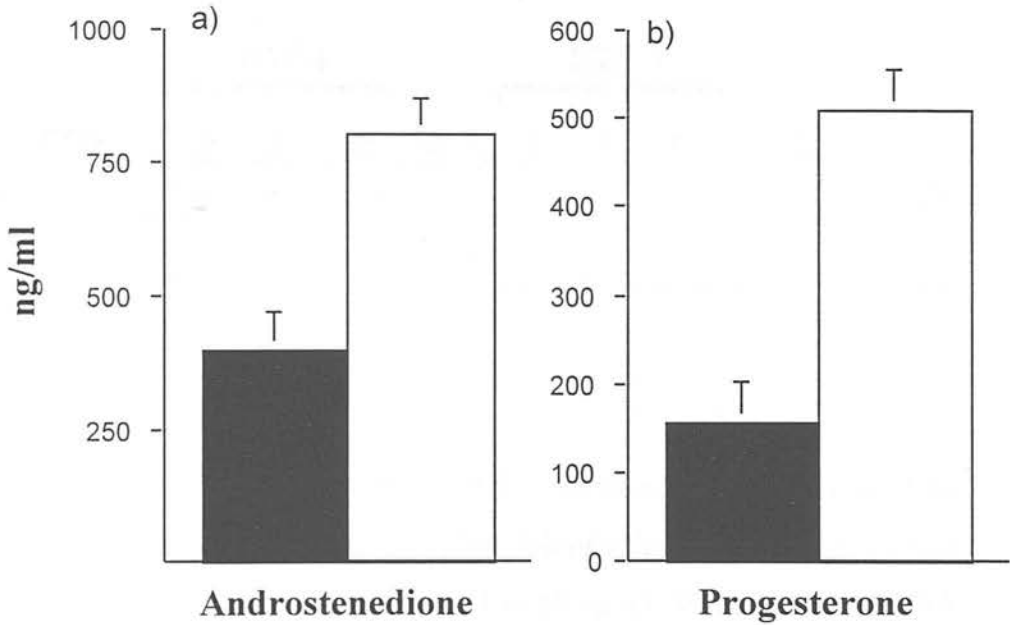


Figure 10.4. Androstenedione (a) and progesterone (b) production by bovine theca cells from medium-sized (4-8 mm) follicles culture for 96 h with 100 ng ml⁻¹ of insulin and 0 (■) or 100 (□) ng ml⁻¹ LH. Values are least square means \pm SEM.

10.4.2 IGF mRNA EXPRESSION.

Reverse transcriptase-polymerase chain reaction (PCR) demonstrated that both IGF-I and -II mRNA were expressed by the liver (Figures 10.5-10.7), thus serving as a positive control. Before culture, IGF-I mRNA was expressed in theca cells (Figure 10.6), but not in granulosa cells (Figures 10.5, 10.6). In contrast, IGF-II mRNA was

expressed in freshly isolated granulosa and theca cells (Figure 10.5, 10.7). After 96 hours of culture, expression of IGF-II mRNA was maintained in both granulosa (Figure 10.5, 10.7) and theca cells (Figure 10.7), but there was no apparent effect of varying the dose of FSH or LH on IGF-II mRNA in either cell type. At the end of culture (96 h), theca cells expressed IGF-I mRNA (Figure 10.6) and its expression was not affected by LH. However, IGF-I mRNA expression in granulosa cells was negligible and appeared to be enhanced by a luteinising dose of FSH (50 ng ml⁻¹; Figure 10.5), but not LH (Figure 10.6).

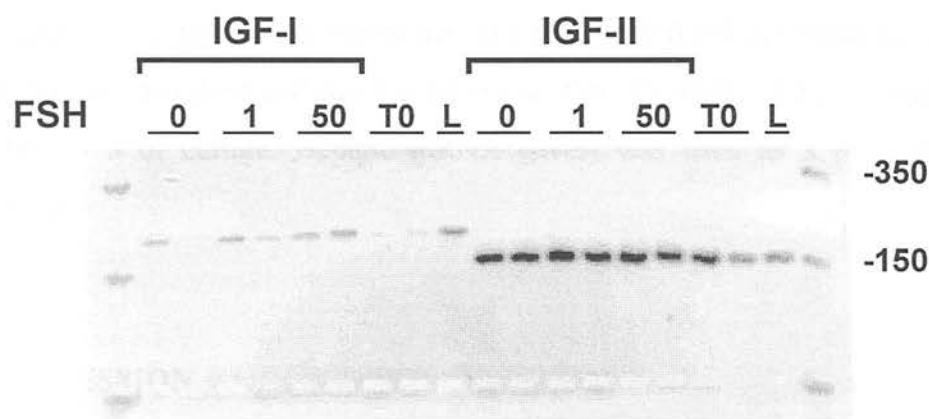


Figure 10.5. IGF-I and IGF-II mRNA expression by bovine granulosa cells at the start of culture and after 96h of culture. Medium contained 10 ng ml⁻¹ of insulin and either 0, 1 or 50 ng ml⁻¹ FSH. Hepatic mRNA (L) was used as a positive control

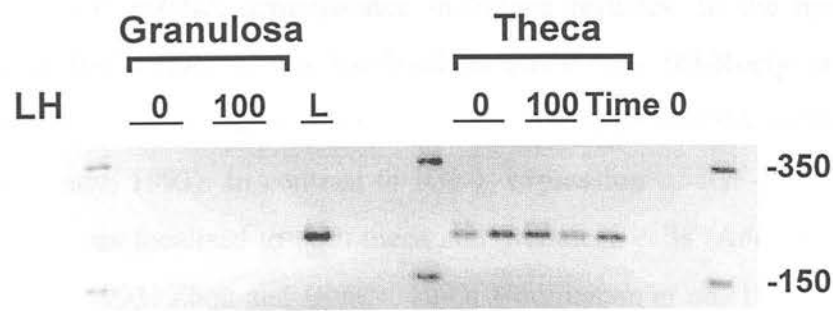


Figure 10.6. Effect of 0or 100 ng ml⁻¹ of LH on IGF-I mRNA expression by bovine granulosa and theca cells at the start of culture (Time 0) and after 96 h of culture. Hepatic mRNA (L) was used as a positive control.



Figure 10.7. Effect of 0 or 100 ng ml⁻¹ of LH on IGF-II mRNA expression by bovine granulosa and theca cells at the start of culture (Time 0) and after 96 h of culture. Hepatic mRNA (liver) was used as a positive control.

10.5 DISCUSSION

The results of this study demonstrate the compartmentalised expression of IGF in the bovine ovary. IGF-I was expressed in freshly isolated cells from the theca layer of the follicle, whilst IGF-II was expressed in both the theca and granulosa cells. The results of these studies in cattle also show a striking similarity with the pattern of expression of IGF mRNA demonstrated in human follicles. In the human ovary, expression of IGF-I mRNA was localised to theca cells (El-Roeiy *et al.*, 1993; Voutilainen *et al.*, 1996), although others did not find IGF-I mRNA within the ovary (Zhou and Bondy, 1993). In contrast to IGF-I, expression of IGF-II mRNA in the human follicle was localised to both theca and granulosa cells (Adashi *et al.*, 1991; El-Roeiy *et al.*, 1993; Zhou and Bondy, 1993; Voutilainen *et al.*, 1996) as observed in this study.

The pattern of IGF-I and -II mRNA expression in the two monovulatory species discussed above (human and bovine), differs from that found for polyovulatory species, where IGF-I mRNA expression is found solely in granulosa cells (pigs:

Samaras *et al.*, 1996; Zhou *et al.*, 1996; rats: Oliver *et al.*, 1989; Adashi *et al.*, 1991; Botero *et al.*, 1994, Hernandez, 1995) and IGF-II mRNA is either not expressed in the follicle (rat: Oliver *et al.*, 1989; Adashi *et al.*, 1991; Botero *et al.*, 1994, Hernandez, 1995) or is expressed in both granulosa and theca cells (pigs: Zhou *et al.*, 1996). In ruminants, however, the pattern of expression of IGF in the follicle is not clear, with contradictory results being reported for sheep (Leewenberg *et al.*, 1995; Perks *et al.*, 1995). In cattle, a recent study localised IGF-II to theca, but not to granulosa cells. Nonetheless, the results of the present study support the paracrine action of IGF-I on granulosa cells and the possible autocrine and paracrine action of IGF-II on granulosa and theca cells. Therefore, the differential expression of IGF-I and -II between mono- and poly-ovulatory species may highlight important differences in the intraovarian regulatory mechanisms controlling the number of follicles that ovulate.

LH increased androstenedione and progesterone production by theca cells, as shown for ovine thecal cells in similar culture conditions (Campbell *et al.*, unpublished data). The optimal dose of LH for steroid production and proliferation of theca cells is around 0.1 to 1 ng/ml whereas doses of LH above 10 ng/ml have a luteinising effect. Therefore, a degree of luteinisation may have occurred in theca cells in culture. In granulosa cells, as reported previously (see Chapter 6) 1 ng/ml FSH stimulated oestradiol production and did not cause luteinisation of the granulosa cells (see Chapter 6). However, as expected, FSH at doses of 100 ng/ml provoked cell luteinisation, with increased progesterone production and a decline in oestradiol production. Addition of LH to granulosa cells stimulated both oestradiol and progesterone production, but despite the high doses employed (100 ng/ml), unlike FSH, LH did not induce luteinisation. The reason for this is perhaps the low levels (or lack) of LH receptor in the granulosa cells. Granulosa cells for this study were obtained from follicles <8mm in diameter, and it is at about 7-8mm in diameter that follicles acquire LH receptors and LH responsiveness (Xu *et al.*, 1995b). Further studies will be needed to determine the regulation of LH receptor expression and LH responsiveness of maturing granulosa cells *in vitro*.

Regardless of the doses of FSH or LH applied to granulosa or theca cell cultures, IGF-II mRNA expression was maintained in both cell types. These results are in agreement with previous studies, where IGF-II mRNA expression within the follicle, was reported in follicles of all size categories including atretic follicles (Zhou and Bondy, 1993; Voutilainen *et al.*, 1996; Zhou *et al.*, 1996). IGF-II mRNA expression has also been detected in luteinised follicular cells and in corpora lutea (Perks *et al.*, 1995).

The expression of IGF-I mRNA in theca cells was present both before and after culture, with no apparent change in expression with the addition of LH. The regulation of IGF-I mRNA in the thecal cell compartment has not been investigated, although one study reported that IGF-I mRNA was present in thecal cells of all follicles < 8mm diameter, but not in the two large dominant follicles of their study (El Roeiy *et al.*, 1993). In contrast, expression of IGF-I mRNA in granulosa cells appeared to increase with time in culture and with dose of FSH, but not LH. IGF-I mRNA levels in granulosa cells are reported to be upregulated by FSH, diethylstilbestrol and GH (Hammond *et al.*, 1991; Adashi, 1992a; Hernandez, 1995; Monget and Monniaux, 1995; Samaras *et al.*, 1996). However, these studies, on the regulation of IGF-I transcripts, were conducted with either rat or porcine granulosa cells which are known to normally express IGF-I. In the current study, the increased expression of IGF-I mRNA in granulosa cells after culture, particularly at the high dose of FSH (50 ng/ml), was accompanied by a decline in oestradiol production but increased progesterone production, indicating that expression of IGF-I mRNA may be linked to luteinisation of the granulosa cells. These observations agree with the results of Wathes *et al.* (1995), who reported immunoreactive IGF-I production by luteinised bovine granulosa cells in culture, and with the finding of IGF-I mRNA expression in bovine granulosa cells after stimulating the ovaries with superovulatory doses of FSH (Spicer *et al.*, 1993b). In addition, the corpus luteum is a site of IGF-I mRNA expression (Einspanier *et al.*, 1990; Perks *et al.*, 1995; Gadsby *et al.*, 1996). Nevertheless, although IGF-I mRNA in human granulosa cells has not been identified by direct methods (*in situ* hybridisation, northern blot analysis, solution hybridisation RNase protection assay), it has been identified after PCR amplification

(Voutilainen *et al.*, 1996). Granulosa cells originating from a single pool expressed IGF-I mRNA after culture, but not before culture, indicating that changes in transcription occurred following the differentiation of the granulosa cells in culture. We previously showed that granulosa cells did not produce detectable amounts of IGF-I when treated with 0 to 10 ng/ml of FSH. Also that FSH, at the doses tested, stimulated oestradiol and progesterone production by granulosa cells in a dose-dependent manner, but did not provoke luteinisation after 144 hours of culture (see Chapter 6). In the present study, cells were cultured for 96 hours only. Hence the time in culture, with the doses of FSH employed, may not have been long enough to allow luteinisation of the granulosa cells, and therefore IGF-I production would not be detected. Similar results have been obtained by Wathes *et al.* (1995) for sheep. The concentration of IGF-I in follicular fluid correlates positively with circulating concentrations of IGF-I, and peripheral concentrations of IGF-I were similar to those found in the ovarian vein (Spicer *et al.*, 1993a). Therefore, the ovary does not seem to be a major source of circulating IGF-I. However, the amount of IGF-I produced within the ovary does not need to be high if it acts in an autocrine or paracrine fashion. In addition, bovine granulosa cells are highly sensitive to IGF and stimulation of maximum oestradiol and proliferative responses of the cells *in vitro* requires concentrations 10 to 100 fold lower than those in the circulation (Campbell *et al.*, 1995; 1996; see Chapter 6). Thus, only a small amount of bioactive IGF would be required within the follicle, particularly since excess IGF-I, of either hormonal or local origin, is regulated by the local production of IGFBP (Armstrong *et al.*, 1996b;c). Indeed, IGF-binding proteins in the follicular fluid vary according to the developmental status of the follicle (Monget *et al.*, 1993). Furthermore, the proportional amount of the different IGFBPs also change with follicular development. IGFBP-2, -3, -4, and -5 were found in follicular fluid of follicles <5mm diameter (De La Sota *et al.*, 1996; Funston *et al.*, 1996). Amounts of the small molecular weight IGFBPs (IGFBP-2, -4 and -5) declined with follicle growth (De La Sota *et al.*, 1996; Funston *et al.*, 1996; Stewart *et al.*, 1996) and with granulosa cell maturation *in vitro* (Armstrong *et al.*, 1996b), while IGFBP-3 levels were unchanged.

These changes in both the quantity and quality of IGFBPs within the follicle may control the local availability of systemic or locally-produced IGFs.

From the results of this study, it is concluded that IGF-II mRNA is expressed in both the granulosa and thecal cell compartments of the bovine ovary, and its expression is independent of follicle development and gonadotrophin stimulation. In contrast, IGF-I mRNA is expressed only by thecal cells and by luteinised granulosa cells, in a developmentally-regulated manner.

11. GENERAL DISCUSSION

This work has investigated the role of metabolic hormones in mediating nutritional effects on bovine follicular development. Both *in vivo* and *in vitro* approaches were used to tackle this problem.

In vivo, the effects of dietary intake (see Chapter 4) and of high energy demand (*e.g.* early lactation; see Chapter 5) on hormonal metabolic milieu and follicular development were investigated. In Chapter 4, it was shown that enhanced dietary intake in heifers increased the number of small (<4mm diameter) follicles present in the ovaries, and that this effect disappeared when the nutritional treatment was withdrawn. In Chapter 5, although the metabolic status of the animals studied varied considerably from the heifers described in Chapter 4, the results were consistent in that improved metabolic status (positive changes in body weight) of the animals correlated with a higher number of follicles in the ovary. From both studies (see Chapters 4 and 5), it appears that circulating GH was not directly involved in stimulating follicular development. However, serum insulin was correlated with the increase in small follicle number (see Chapter 4 and 5) and with earlier ovulation postpartum (Chapter 5).

In Chapter 4, no differences in FSH concentrations were found between dietary treatments. Thus, changes in follicle number seem to be independent of changes in circulating FSH. In dairy cattle, no differences in follicle number or in FSH levels were seen between high or low milk yielders. However, the first ovulation postpartum occurred earlier in animals selected for low milk yield. Reduced LH pulse frequency in the postpartum period is the main cause of ovulatory failure and is associated with low energy balance (Butler and Smith, 1989; Canfield and Butler, 1990; Robinson 1996). However, LH was not measured in this study. Hence, in the first instance, the explanation for the later ovulation in cows selected for high milk production would seem to be that their energy balance is more negative than cows of lower genetic merit. However, there were no differences in milk production or in predicted changes in body weight during the period of study. Therefore, no direct evidence exists to support a lower energy balance of animals in the high milk yield

selection line. Nonetheless, the higher levels of GH and BOHB in selected cows suggests a lower energy balance, but since the genetically superior animals show a greater response in GH and BOHB to similar metabolic stresses (Lovendahl *et al.*, 1991; Woolliams *et al.*, 1992; Xing *et al.*, 1993; Barnes *et al.*, 1985) these measures cannot be considered as good indicators of metabolic status. The role of insulin and IGF-I was also investigated in these studies.

An extensive evaluation of the methods available for the extraction of IGF-I before radioimmunoanalysis was conducted in Chapter 3. Several interesting observations were made. a) Most IGF extraction protocols were inefficient in completely removing IGFBPs. b) The displacement curve of isolated IGFBP was parallel to the IGF-I standard; hence parallelism between the IGF extract and the standard cannot be used to demonstrate the complete removal of IGFBPs from the sample. c) Residual IGFBPs interfere in the IGF-I RIA giving inaccurate results. d) IGFBP interference could result in either overestimation or underestimation of total IGF-I contained in the sample and depended on the IGF-I to IGFBP ratio. If the IGF-I to IGFBP ratio was <1 the results will be overestimated. If however the ratio was >1 , total IGF-I will be underestimated. e) Finally, acid gel HPLC was the only procedure capable of eliminating the interference of IGFBPs completely.

Acid gel HPLC was used to extract total IGF-I from samples in heifers under different nutritional treatments (see Chapter 4). It was found that total IGF-I was 2-3 fold higher than previously reported values for cattle (Spicer *et al.*, 1992b; Armstrong *et al.*, 1993; Gong *et al.*, 1993a). However, no differences in total IGF-I concentrations were found between dietary treatments. These results contrast with the diet-induced alterations in IGF-I levels found by others (Breier *et al.*, 1986; Armstrong *et al.*, 1993; Clarke *et al.*, 1993; Yambayamba *et al.*, 1996) and may be explained by the protocol used to extract IGF-I before assay. Therefore, if nutritional status also affects circulating amounts and species of IGFBPs (Pell and Bates, 1990; Hodgkinson *et al.*, 1991; McGuire *et al.*, 1992; Pao *et al.*, 1993; Takenaka *et al.*, 1996), the bioavailability of IGF-I would be also altered. In this scenario a study of IGF-I concentrations will be incomplete if changes in IGFBPs are not considered.

The *in vivo* studies highlighted important associations between metabolic status, metabolic hormones and follicular development. However, despite these associations they do not prove a direct involvement of the metabolic hormones on follicular development. To study the direct effects of metabolic hormones and their interactions with gonadotrophins, a series of *in vitro* studies were carried out. A serum-free granulosa cell culture system was developed for cattle (see Chapter 6), based on the culture system developed for ovine granulosa cells (Campbell *et al.*, 1996a). In this culture system, bovine granulosa cells from small, medium-sized and large follicles maintained oestradiol production and proliferative responses to physiological concentrations of FSH. Furthermore, undifferentiated granulosa cells from small follicles matured *in vitro* and increased their oestradiol production with time. In addition, cell organisation *in vitro* was characterised by the formation of clumps consisting of spherical cells (see Chapter 6). Cells within these cell aggregates were tightly joined and contained extensive gap junctions between cells (see Chapter 7). Moreover, organelle characteristics of the cells within the clumps were typical of non-luteinised granulosa cells within the follicular wall (see Chapter 7). These results supported previous results linking granulosa cell structure and function (Amsterdam and Rotsmensch, 1987).

In culture, IGF-I stimulated granulosa cell proliferation and oestradiol production (see Chapter 6). However, granulosa cell stimulation by the IGF-I analogue LR3-IGF-I (which does not bind to IGFBPs) was more potent than the native hormone. This supports the notion that bovine granulosa cells also secrete IGFBPs (Armstrong *et al.*, 1996 b,c). The inclusion of LR3-IGF-I into the culture stimulated both cell proliferation and oestradiol production, even in the absence of FSH, demonstrating the gonadotrophic-like actions of this growth factor and its potential role in follicular development. Insulin also stimulated both cell proliferation and oestradiol production in a dose-responsive manner, reaching a maximum at doses of 50 ng/ml (see Chapter 6). At these high insulin concentrations, FSH did not stimulate cell proliferation or oestradiol production, which may explain the failure of FSH to stimulate granulosa cells in cultures containing high insulin concentrations (Saumande, 1991; Langhout *et al.*, 1991; Berndtson *et al.*, 1995).

Despite the proliferative effect of insulin and IGF-I, they were unable to stimulate proliferation of granulosa cells from subordinate follicles, recovered on day 7 of the oestrous cycle (see Chapter 8), in comparison with cells from follicles collected on day 3 of the cycle. *i.e.* before the establishment of the dominant follicle. However, although oestradiol production by cells from day 7 medium-sized follicles was lower in the first 48 h of culture, oestradiol production at 144 h of culture did not differ between cells recovered on either day 3 or day 7 of the oestrous cycle. These results contradict the proposed mechanism of action for insulin in increasing follicle development by rescuing follicles from atresia (Downing and Scaramuzzi, 1991; Matamoros *et al.*, 1990; 1991). Our *in vitro* data suggest that recruitment of new follicles (rather than rescue from follicular atresia) is the most likely mechanism by which nutrition can enhance antral follicle number.

If the increase in follicle number is not due to rescue of follicles from atresia, follicle growth must be stimulated in follicles from the preantral stage. Little is known about which factors stimulate and sustain preantral follicle growth. It is assumed that at this early stage of development, follicle growth is gonadotrophin-independent. However, similar to the situation for granulosa cells (see Chapter 6), a model in which factors affecting preantral follicle growth can be studied is not available to date, and attempts to develop a preantral follicle culture failed because preantral follicles lost their three-dimensional architecture, and cells plated down leaving a denuded oocyte after few days in culture.

In Chapter 9, a preantral follicle culture is described where preantral follicles in long-term culture maintain their three-dimensional architecture, grow and finally develop antral spaces. In this culture system, the effects of FSH, IGF-I and EGF alone or in combination enhanced follicular growth. Similarly, the probability of antrum formation was greater in treated than in untreated follicles. However, no differences in growth were seen between follicles treated with FSH, IGF-I or EGF, or when FSH was combined with IGF-I or EGF. Hence, our results support a role for IGF-I and EGF, and equally FSH in early follicular development. In addition, in all instances, preantral follicles grew in culture even in the absence of added growth factors or

FSH. These results, together with those of Wandji *et al.* (1996) and Braw-Tal and Yossefi (1997) who observed preantral follicle growth in isolated ovarian cortical slices, suggest the probable presence of a preantral follicle growth inhibitor acting within the ovary *in vivo*. The use of this culture system will assist the study of the regulation of early follicular growth and antrum formation.

Finally, the putative production and gonadotrophic regulation of IGF-I by bovine granulosa cells was investigated. Non-luteinised granulosa cells produced immunoreactive IGF-I (see Chapter 3). However, when IGF-II was added to block IGFBPs, IGF-I immunoreactivity decreased below the detection limit of the assay (see Chapter 3). FSH (up to 10 ng/ml) and insulin did not induce detectable amounts of IGF-I production by granulosa cells (see Chapter 10). When investigating the expression of IGF-I and -II mRNA from granulosa and theca cells, IGF-I mRNA was expressed in fresh theca, but not granulosa cells. After culture, IGF-I mRNA expression was maintained in theca cells and appeared in granulosa cells cultured with a luteinising dose of FSH (50 ng/ml). IGF-II was expressed in fresh theca and granulosa cells both before and after culture, regardless of the dose of gonadotrophin used. These results help to clarify the controversy surrounding IGF-I mRNA expression in the ovary of ruminant species (Spicer *et al.*, 1993b; Leewenberg *et al.*, 1995; Perks *et al.*, 1995) and support an autocrine action of IGF-II on granulosa and theca cells, and a paracrine action of IGF-I on granulosa cells. Interestingly, the pattern of expression of IGF-I and -II mRNA in the bovine ovary is identical to that observed in the human ovary, further supporting the cow as an animal model for human reproduction.

In summary, these studies demonstrated that dietary manipulations can affect follicular recruitment in cattle, and that the numbers of antral follicles in the ovary are in direct relation to the metabolic status of the animal. From these studies of animals in moderate to good body condition, the metabolic mediators between nutrition and follicular development appear to act directly at the ovarian level, since no changes were seen in FSH concentrations due to nutritional treatment (see Chapter 4) or genetic line (see Chapter 5). In addition, *in vitro* studies demonstrated

that insulin and IGF-I stimulate both the proliferation and differentiation of bovine granulosa cells. Furthermore, it was shown that the granulosa cells are not the site of IGF-I production. However, theca and perhaps luteinised granulosa cells of the dominant preovulatory follicle did express IGF-I mRNA. Nevertheless, peripheral and intraovarian regulation of circulating IGF-I action, through changes in IGFBP levels warrants further investigation.

The culture systems developed during the work of this thesis should also provide a valuable tool for future research investigating the mechanisms that control follicular development and granulosa cell differentiation and function, in particular the relationship between nutrition and reproduction.

12. BIBLIOGRAPHY

- Adams GP, Kot K, Smith CA and Ginther OJ. (1993). Selection of a dominant follicle and suppression of follicular growth in heifers. *Anim. Reprod. Sci.* **30**, 259-271.
- Adams GP, Evans ACO and Rawlings NC. (1994). Follicle waves and circulating gonadotrophins in 8-month-old prepubertal heifers. *J. Reprod. Fertil.* **100**, 27-33.
- Adams NR, Briegel JR, Rigby RDG, Sanders MR and Hoskinson RM. (1996). Responses of sheep to annual cycles in nutrition. 1. Role of endogenous growth hormone during undernutrition. *Anim. Sci.* **62**, 279-286.
- Adashi EY, Resnick CE, Hurwitz A, Ricciarelli E, Hernandez ER, Roberts CT, LeRoith D and Rosenfeld R. (1991). Insulin-like growth factors: the ovarian connection. *Hum. Reprod.* **6**, 1213-1219.
- Adashi EY and Rohan RM. (1992). Intraovarian regulation. Peptidergic signaling systems. *Trends Endocrinol. Metabol.* **3**, 243-248.
- Adashi EY. (1992a). Intraovarian regulation: the IGF-I example. *Reprod. Fertil. Dev.* **4**, 497-504.
- Adashi EY. (1992b). Intraovarian peptides: stimulators and inhibitors of follicular growth and differentiation. *Endocrinol. Metabol. Clin. North. Amer.* **21**, 1-17.
- Adashi EY. (1995). Insulin-like growth factors as determinants of follicular fate. *J. Soc. Gynecol. Invest.* **2**, 721-726.
- Albecia JA, Rhind SM, Bramley TA and McMillen SR. (1995). Steroid production and LH receptor concentrations of ovarian follicles and corpora lutea and associated rates of ova wastage in ewes given high and low levels of food intake before and after mating. *Anim. Sci.* **61**, 57-62.
- Aleksiev AI, Peeva T, Vasilev M and Folikhronov O. (1988). Effect of season of calving on reproductive performance and productivity of buffalo cows. *Zhivotnov'd Nauki* **25**, 3-10.
- Alpizar E and Spicer LJ. (1993). Effects of interleukin-6 on proliferation and follicle-stimulating hormone-induced estradiol production by bovine granulosa cells in-vitro: Dependence on Size of follicle. *Biol. Reprod.* **49**, 38-43.
- Amaral-Phillips DM, McGilliard AD, Lindberg GL, Veenhuizen JJ and Young JW. (1993). Effects of decreased availability of glucose for dairy cows. *J. Dairy Sci.* **76**, 752-761.
- Amselgruber W, Sinowatz F, Schams D and Skottner A. (1994). Immunohistochemical aspects of insulin-like growth factor I and II in the bovine corpus luteum. *J. Reprod. Fertil.* **101**, 445-451.
- Amsterdam A and Rotmensch S. (1987). Structure-function relationships during granulosa cell differentiation. *Endocr. Rev.* **8**, 309-337.

- Armstrong DG, Duclos MJ and Goddard C. (1990). Biological activity of insulin-like growth factor-I purified from chicken serum. *Dom. Anim. Endocrinol.* **7**, 383-393.
- Armstrong JD, Cohick WS, Harvey RW, Heimer EP and Campbell RM. (1993). Effect of feed restriction on serum somatotropin, insulin-like growth factor-1 (IGF-1) and binding proteins in cyclic heifers actively immunized against growth hormone releasing factor. *Dom. Anim. Endocrinol.* **10**, 315-324.
- Armstrong DT, Xia P, Gannes Gd, Tekpetey FR and Khamisi F. (1996a). Differential effects of insulin-like growth factor-I and follicle-stimulating hormone on proliferation and differentiation of bovine cumulus cells and granulosa cells. *Biol. Reprod.* **54**, 331-338.
- Armstrong DG, Hogg CO, Campbell BK and Webb R. (1996b). Insulin-like growth factor (IGF)-binding protein production by primary cultures of ovine granulosa and theca cells. The effects of IGF-I, gonadotropin, and follicle size. *Biol. Reprod.* **55**, 1163-1171.
- Armstrong DG, Gutierrez CG, Hogg CO and Campbell BK. (1996c). The expression of insulin-like growth factor binding proteins (IGFBP) mRNA in primary cultures of bovine granulosa cells. *J. Reprod. Fertil. Abstr. Series* **17**, Abstract 30.
- Armstrong DG, Baxter G, Gutierrez CG, Woad KJ, Sinclair KD, Robinson JJ, McEnvoy TG and Webb R. (1997). Ovarian IGF-II mRNA expression in cattle fed different energy and protein diets. *J. Reprod. Fertil. Abstr. Series* **19**, In press.
- Baird DT and McNeilly AS. (1981). Gonadotrophic control of follicular development and function during the oestrous cycle of the ewe. *J. Reprod. Fertil. Suppl.* **30**, 119-133.
- Baird DT, Campbell BK, Mann GF and McNeilly AS. (1991). Gonadotrophic control of follicle growth in the ewe. *J. Reprod. Fertil. Suppl.* **43**, 125-138.
- Baker J, Liu JP, Robertson EJ and Efstratiadis A. (1993). Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* **120**, 73-82.
- Bang P. (1994). Valid measurements of total IGF concentrations in biological fluids. Recommendations from the 3rd International Symposium on Insulin-like growth factors. *J. Endocrinol.* **143**, C1-C2.
- Bao B, Garverick HA, Smith GW, Smith MF, Salfen BE and Youngquist RS. (1997). Expression of messenger RNA encoding 3 β -hydroxysteroid dehydrogenase/d4,d5 isomerase during recruitment and selection of bovine ovarian follicles: identification of dominant follicles by expression of 3 β -HSD mRNA within the granulosa cell layer. *Biol. Reprod.* **In press**.
- Barash IA, Cheung CC, Weigle DS, Ren H, Kabigting EB, Kuijper JL, Clifton DK and Steiner RA. (1996). Leptin is a metabolic signal to the reproductive system. *Endocrinology* **137**, 3144-3147.
- Barnard R and Waters MJ. (1997). The serum growth hormone binding protein: pregnant with possibilities. *J. Endocrinol.* **153**, 1-14.

- Barnes MA, Kazmer GW, Akers RM and Pearson RE. (1985). Influence of selection for milk yield on endogenous hormones and metabolites in holstein heifers and cows. *J. Anim. Sci.* **60**, 271-284.
- Bauman DE. (1992). Bovine somatotropin: Review of an emerging animal technology. *J. Dairy Sci.* **75**, 3432-3451.
- Baxter RC and Martin JL. (1989). Binding proteins for the insulin-like growth factors: structure, regulation and function. *Prog. Growth Fact. Res* **1**, 49-68.
- Baxter G, O'Shea T, Campbell B and Webb R. (1995). Effects of bovine follicular fluid (bFF) fractions, which delay oestrus in sheep and cattle, on proliferation and steroid production by cultured granulosa cells. *J. Reprod. Fertil. Abstr. series* **15**, Abstr. 189.
- Beam SW and Butler WR. (1997). Energy balance and ovarian follicle development prior to the first ovulation postpartum in dairy cows receiving three levels of dietary fat. *Biol. Reprod.* **56**, 133-142.
- Beard AJ, Savva D, Glencross RG, McLeod BJ and Knight PG. (1989). Treatment of ovariectomized heifers with bovine follicular fluid specifically suppresses pituitary levels of FSH-Beta messenger RNA. *J. Mol. Endocrinol.* **3**, 85-92.
- Berndtson AK, Vincent SE and Fortune JE. (1995). Low and high concentrations of gonadotropins differentially regulate hormone production by theca interna and granulosa cells from bovine preovulatory follicles. *Biol. Reprod* **52**, 1334-1342.
- Besnard N, Pisselet C, Zapf J, Hornebeck W, Monnaiux D and Monget P. (1996). Proteolytic activity is involved in changes in intrafollicular insulin-like growth and atresia of ovine ovarian follicles. *Endocrinology* **137**, 1599-1607.
- Billig H, Chun SY, Eisenhauer K and Hsueh AJW. (1996). Gonadal cell apoptosis: hormone-regulated cell demise. *Human Reprod Update* **2**, 103-117.
- Billington CJ and Levine AS. (1996). Appetite regulation: Shedding new light on obesity. **6**, 920-923.
- Bishop DK, Wettemann RP and Spicer LJ. (1994). Body energy reserves influence the onset of luteal activity after early weaning of beef cows. *J. Anim. Sci.* **72**, 2703-2708.
- Bjersing L. (1978). Maturation, morphology, and endocrine function of the follicular wall in mammals. In *The vertebrate ovary* (ed. Jones), pp. 181-214. Plenum press, New York.
- Blum WF, Ranke MB and Bierich JR. (1988). A specific radioimmunoassay for insulin-like growth factor II: the interference of IGF binding proteins can be blocked by excess IGF-I. **118**, 374-380.
- Blum WF and Breier BH. (1994). Radioimmunoassays for IGFs and IGFBPs. **4**, 11-19.
- Boden G, Chen X, Mozzoli M and Ryan I. (1996). Effect of fasting on serum leptin in normal human subjects. *J. Clin. Endocrinol. Metabol.* **81**, 3419-3423.

- Boland NI, Humpherson PG, Leese HJ and Gosden RG. (1993). Pattern of lactate production and steroidogenesis during growth and maturation of mouse ovarian follicles in vitro. *Biol. Reprod.* **48**, 798-806.
- Botero LF, Roberts CT, Roith DL, Adashi EY and Hernandez ER. (1993). Insulin-like growth factor I gene expression by primary cultures of ovarian cells: Insulin and dexamethasone dependence. **132**, 2703-2708.
- Braw-Tal R and Yossefi S. (1997). Studies in vivo and in vitro on the initiation of follicle growth in the bovine ovary. *J. Reprod. Fertil.* **109**, 165-171.
- Breier BH, Bass JJ, Butler JH and Gluckman PD. (1986). The somatotrophic axis in young steers: influence of nutritional status on pulsatile release of growth hormone and circulating concentrations of insulin-like growth factor 1. *J. Endocrinol.* **111**, 209-215.
- Breier BH, Gallaher BW and Gluckman PD. (1991). Radioimmunoassay for insulin-like growth factor-I: solutions to some potential problems and pitfalls. *J. Endocrinol.* **128**, 347-357.
- Breslow NE and Clayton DG. (1988). Approximate inference in generalized linear mixed models. *J. Am. Stat. Assoc.* **88**, 9-25.
- Bronson FH and Manning J. (1991). The energetic regulation of ovulation: A realistic role for body fat. *Biol. Reprod.* **44**, 945-950.
- Brown W, Dziegielewska K, Foreman R and Saunders N. (1990). The nucleotide and deduced aminoacid sequences of insulin-like growth factor II cDNAs from adult and fetal sheep liver. *Nucleic Acids Res.* **18**, 4614-4614.
- Buccione R, Schroeder AC and Eppig JJ. (1990). Interactions between somatic cells and germ cells throughout mammalian oogenesis. *Biol. Reprod.* **43**, 543-547.
- Bucholtz DC, Vidwans NM, Herbosa CG, Schillo KK and Foster KL. (1996). Metabolic interfaces between growth and reproduction. V. Pulsatile luteinizing hormone secretion is dependent on glucose availability. *Endocrinology* **137**, 601-607.
- Butler WR and Smith RD. (1989). Interrelationship between energy balance and postpartum reproductive function in dairy cattle. *J. Dairy Sci.* **72**, 767-783.
- Calamari L, Bertoni G, Maianti MG and Cappa V. (1989). Sull'utilit  di nuovi parametri ematochimici nella valutazione del profilo metabolico della lattifere. *Zoot. Nutr. Anim.* **15**, 191-210.
- Campbell BK, Picton HM, Mann GE, McNeilly AS and Baird DT. (1991). Effect of steroid- and inhibin-free ovine follicular fluid on ovarian follicles and ovarian hormone secretion. *J. Reprod. Fertil.* **93**, 83-96.
- Campbell BK, Scaramuzzi RJ and Webb R. (1995). Control of antral follicle development and selection in sheep and cattle. *J. Reprod. Fertil. Suppl* **49**, 335-350.
- Campbell BK and Scaramuzzi RJ. (1996). The effect of arterial infusion of human recombinant inhibin and bovine follicular fluid on ovarian follicles and ovarian hormone secretion. *J. Endocrinol.* **148**, 531-540.

- Campbell BK, Scaramuzzi RJ and Webb R. (1996a). Induction and maintenance of oestradiol and immunoreactive inhibin production with FSH by ovine granulosa cells cultured in serum-free media. *J. Reprod. Fertil.* **106**, 7-16.
- Campbell KHS, McWhir J, Ritchie WA and Wilmut I. (1996b). Sheep cloned by nuclear transfer from a cultured cell line. *Nature* **380**, 64-66.
- Campbell BK. (1997). Differential effects of insulin-like growth factor-I (IGF-I) and II (IGF-II) on proliferation and differentiation of cultured sheep granulosa cells. *J. Endocrinol. Suppl.* **152**, 236 (Abstract).
- Canfield RW and Butler WR. (1990). Energy balance and pulsatile LH secretion in early postpartum dairy cattle. *Dom. Anim. Endocrinol.* **7**, 323-330.
- Carriere PD, Harvey D and Cooke GM. (1996). The role of pregnenolone-metabolizing enzymes in the regulation of oestradiol biosynthesis during development of the first wave dominant follicle in the cow. *J. Endocrinol.* **149**, 233-242.
- Carson RS, Findlay JK, Clarke IJ and Burger HG. (1981). Estradiol, testosterone, and androstenedione in ovine follicular fluid during growth and atresia of ovarian follicles. *Biol. Reprod.* **24**, 105-113.
- Caubo B, DeVinna RS and Tonetta SA. (1989). Regulation of steroidogenesis in cultured porcine theca cells by growth factors. *Endocrinology* **125**, 321-326.
- Chehab FF, Lim ME and Lu R. (1996). Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nature Genetics* **12**, 318-320.
- Chehab FF, Mounzih K, Lu R and Lim ME. (1997). Early onset of reproductive function in normal female mice treated with leptin. *Science* **275**, 88-90.
- Chun S, Eisenhauer KM, Minami S, Billig H, Perlas E and Hsueh AJW. (1996). Hormonal regulation of apoptosis in early antral follicles: Follicle-stimulating hormone as a major survival factor. *Endocrinology* **137**, 1447-1456.
- Clark RG and Robinson ICAF. (1996). Up and down the growth hormone cascade. *Cytokine Growth Factor Rev.* **7**, 65-80.
- Clarke IJ, Fletcher TP, Pomares CC, Holmes JHG, Dunshea F, Thomas GB, Tilbrook AJ, Walton PE and Galloway DB. (1993). Effect of high-protein feed supplements on concentrations of growth hormone (GH), insulin-like growth factor-I (IGF-I) and IGF-binding protein-3 in plasma and on the amounts of GH and messenger RNA for GH in the pituitary glands of adult rams. *J. Endocrinol.* **138**, 421-427.
- Cohick WS, Armstrong JD, Withacre MD, Lucy MC, Harvey RW and Campbell RM. (1996). Ovarian expression of insulin-like growth factor-I (IGF-I), IGF binding proteins, and growth hormone (GH) receptor in heifers actively immunized against GH-releasing factor. *Endocrinology* **137**, 1670-1677.
- Cole WJ, Madsen KS, Hintz RL and Collier RJ. (1991). Effect of recombinant-derived bovine somatotropin in reproductive performance of dairy cattle. *Theriogenology* **36**, 573-595.

- Cole NA, Hallford DM and Gallavan R. (1993). Influence of a glucose load in fed or unfed lambs on blood metabolites and hormone patterns. *J. Anim. Sci.* **71**, 765-773.
- Corrie JET, Hunter WM and Macpherson JS. (1981). A strategy for radioimmunoassay of plasma progesterone with the use of homologous site ^{125}I -labelled radioligand. *Clin. Chem.* **27**, 594-599.
- Crozet N, Kanka J, Motlik J and Fulka J. (1986). Nucleolar fine structure and RNA synthesis in bovine oocytes from antral follicles. *Gamete Res.* **14**, 65-73.
- Crozet N. (1989). Nucleolar structure and RNA synthesis in mammalian oocytes. *J. Reprod. Fertil.* **38**, 9-16.
- Daughaday WH, Mariz IK and Blethan SC. (1980). Inhibition of access of bound somatomedin to membrane receptor and immunobinding sites: a comparison of radioreceptor and radioimmunoassay of somatomedin in native and acid-ethanol-extracted serum. *J. Clin. Endocrinol. Metabol.* **51**, 781-796.
- De la Sota RL, Lucy MC, Staples CR and Thatcher WW. (1993). Effects of recombinant bovine somatotropin (Sometribove) on ovarian function in lactating and nonlactating dairy cows. *J. Dairy Sci.* **76**, 1002-1013.
- De la Sota RL, Simmen FA, Diaz T and Thatcher WW. (1996). Insulin-like growth factor system in bovine first-wave dominant and subordinate follicles. *Biol. Reprod.* **55**, 803-812.
- Diamanti-Kandarakis E and Dunaif A. (1996). New perspectives in polycystic ovary syndrome. *Trends Endocrinol. Metabol.* **7**, 267-271.
- Dieguez C and Casanueva FF. (1995). Influence of metabolic substrates and obesity on growth hormone secretion. *Trends Endocrinol. Metabol.* **6**, 55-59.
- Dong J, Albertini DF, Nishimori K, Kumar TR, Naifang L and Matzuk MM. (1996). Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* **383**, 531-535.
- Downing JA and Scaramuzzi RJ. (1991). Nutrient effects on ovulation rate, ovarian function and the secretion of gonadotrophic and metabolic hormones in sheep. *J. Reprod. Fertil. Suppl.* **43**, 209-227.
- Downing JA, Joss J and Scaramuzzi RJ. (1995). Ovulation rate and the concentrations of gonadotrophins and metabolic hormones in ewes infused with glucose during the late luteal phase of the oestrous cycle. *J. Endocrinol.* **146**, 403-410.
- Downing JA and Scaramuzzi RJ. (1997). The effect of the infusion of insulin during the luteal phase of the estrous cycle on the ovulation rate and on plasma concentrations of LH, FSH and glucose in ewes. *Theriogenology* **47**, 747-759.
- Driancourt MA, Fry RC, Clarke IJ and Cahill LP. (1987). Follicular growth and regression during the 8 days after hypophysectomy in sheep. *J. Reprod. Fertil.* **79**, 635-641.

- Driancourt MA. (1991). Follicular dynamics in sheep and cattle. *Theriogenology* **35**, 55-79.
- Drop SLS, Brinkman A, Kortleve DJ, Groffen CAH, Schuller A and Zwarthoff EC. (1991). The evolution of the insulin-like growth factor binding protein family. In *Modern concepts of insulin-like growth factors* (ed. Spencer), pp. 311-328. Elsevier Science Publishing Co., Inc.
- Dufour J, Cahill LP and Mauleon P. (1979). Short- and long-term effects of hypophysectomy and unilateral ovariectomy on ovarian follicular populations in sheep. *J. Reprod. Fertil.* **57**, 301-309.
- Duke RC, Ojcius DM and Young JDE. (1996). Cell suicide in health and disease. *Sci. Am.* **Dec.**, 48-55.
- Duleba AJ, Pawelczyk LA, Yuen BH and Moon YS. (1993). Insulin actions on ovarian steroidogenesis are not modulated by metformin. *Human Reprod.* **8**, 1194-1198.
- Dunn TG and Moss GE. (1992). Effects of nutrient deficiencies and excesses on reproductive efficiency of livestock. *J. Anim. Sci.* **70**, 1580-1593.
- Echternkamp SE, Spicer LJ, Gregory KE, Canning SF and Hammond JM. (1990). Concentrations of insulin-like growth factor-1 in blood and ovarian follicular fluid of cattle selected for twins. *Biol. Reprod.* **43**, 8-14.
- Edwards JL, Hughey TC, Moore AB and Cox NM. (1996). Depletion of insulin in streptozocin-induced-diabetic pigs alters estradiol, insulin-like growth factor (IGF)-I and IGF binding proteins in cultured ovarian follicles. *Biol. Reprod.* **55**, 775-781.
- Einspanier R, Miyamoto A, Schams D, Mýller M and Brem G. (1990). Tissue concentration, mRNA expression and stimulation of IGF-I in luteal tissue during the oestrous cycle and pregnancy of cows. *J. Reprod. Fertil.* **90**, 439-445.
- El-Roeiy A, Chen X, Roberts VJ, LeRoith D, Roberts CT and Yen SSC. (1993). Expression of insulin-like growth factor-I (IGF-I) and IGF-II and the IGF-I, IGF-II, and insulin receptor genes and localization of the gene products in the human ovary. *J. Clin. Endocrinol. Metabol.* **77**, 1411-1418.
- Elsasser TH, Rumsey TS and Hammond AC. (1989). Influence of diet on basal and growth hormone-stimulated plasma concentrations of IGF-1 in beef cattle. *J. Anim. Sci.* **67**, 128-141.
- England BG, Dahmer MK and Webb R. (1981). Relationships between follicular size and antral fluid steroid concentrations at three stages of the estrous cycle in the ewe. *Biol. Reprod.* **24**, 1068-1075.
- Enright WJ, Chapin LT, Moseley WM, Zinn SA, Kamdar MB, Krabill LF and Tucker HA. (1989). Effects of infusions of various doses of bovine growth hormone-releasing factor on blood hormones and metabolites in lactating Holstein cows. *J. Endocrinol* **122**, 671-679.

- Enright WJ, Spicer LJ, Prendiville DJ, Murphy MG and Campbell RM. (1994). Interaction between dietary intake and ovariectomy on concentrations of insulin-like growth factor-I, GH and LH in plasma of heifers. *Theriogenology* **41**, 1231-1240.
- Enriquez BA, Galina CS, Navarro RR and Gutierrez C. (1993). Estimacion de la epoca mas propicia para un empadre estacional en ganado cebu bajo condiciones de tropico humedo. *Avan. Invest. Agropec.* **2**, 101-114.
- Eppig JJ and Downs SM. (1984). Chemical signals that regulate mammalian oocyte maturation. *Biol. Reprod.* **30**, 1-11.
- Eppig JJ and Downs SM. (1987). The effect of hypoxanthine on mouse oocyte growth and development *in vitro*: maintainance of meiotic arrest and gonadotropin-induced oocyte maturation. *Dev. Biol.* **119**, 313-321.
- Eppig JJ and Schroeder AC. (1989). Capacity of the mouse oocyte from preantral follicles to undergo embryogenesis and development to live young after growth, maturation, and fertilization *in vitro*. *Biol. Reprod.* **41**, 268-276.
- Eppig JJ. (1991). Intercommunication between mammalian oocytes and companion somatic cells. *BioEssays* **13**, 569-574.
- Eppig JJ and O'Brien MJ. (1996). Development *in vitro* of mouse oocytes from primordial follicles. *Biol. Reprod.* **54**, 197-207.
- Esteban E, Kass PH, Weaver LD, Rowe JD, Holmberg CA, Franti CE and Trout HF. (1994). Interval from calving to conception in high producing dairy cows treated with recombinant bovine somatotopin. *J. Dairy Sci.* **77**, 2549-2561.
- Estienne MJ, Schillo KK, Hileman SM, Green MA, Hayes SH and Boling J. (1990). Effects of free fatty acids on luteinizing hormone and growth hormone secretions in ovariectomized lambs. *Endocrinology* **126**, 1934-1940.
- Fair T, Hyttel P and Greve T. (1995). Bovine oocyte diameter in relation to maturational competence and transcriptional activity. *Mol. Reprod. Develop.* **42**, 437-442.
- Falcieri E, Gobbi P, Zamai L and Vitale M. (1994). Ultrastructural features of apoptosis. *Scanning Microsc.* **8**, 653-666.
- Filicori M. (1993). Evidence against the utility of growth hormone in the enhancement of ovulation. In *The somatotrophic axis and the reproductive process in health and disease*, pp. 29. Sero Symposium, Baltimore, Maryland.
- Fortune JE, Sirois J, Turzillo AM and Lavoie M. (1991). Follicle selection in domestic ruminants. *J. Reprod. Fertil. Suppl.* **43**, 187-198.
- Fortune JE. (1994). Ovarian follicular growth and development in mammals. *Biol. Reprod.* **50**, 225-232.
- Foster DL, Ebling FJP, Micka AF, Vannerson LA, Bucholtz DC, Wood RI, Suttie JM and Fenner DE. (1989). Metabolic interfaces between growth and reproduction. I. Nutritional modulation of gonadotropin, prolactin, and growth hormone secretion in the growth-limited female lamb. *Endocrinology* **125**, 342-350.

- Fotsis T, Murphy C and Gannon F. (1990). Nucleotide sequence of the bovine insulin-like growth factor I (IGF-I) and its IGF-IA precursor. *Nucleic Acids Res.* **18**, 676-676.
- Frey RS, Hathaway MR and Dayton WR. (1994). Comparison of the effectiveness of various procedures for reducing or eliminating insulin-like growth factor-binding protein interference with insulin-like growth factor-I radioimmunoassays on porcine sera. *J. Endocrinol.* **140**, 229-237.
- Friedman JM. (1997). The alphabet of weight control. *Nature* **385**, 119-120.
- Frisch RE and McArthur JW. (1974). Menstrual cycles: Fatness as a determinant of minimum weight for height necessary for their maintenance and onset. *Science* **185**, 949-951.
- Fulghesu AM, Villa P, Pavona V, Guido M, Apa R, Caruso A, Lanzone A, Rossodivita A and Mancuso S. (1997). The impact of insulin secretion on the ovarian response to exogenous gonadotropins in polycystic ovary syndrome. *J. Clin. Endocrinol. Metabol.* **82**, 644-648.
- Funston RN, Moss GE and Roberts J. (1995). Insulin-like growth factor-I (IGF-I) and IGF-Binding proteins in bovine sera and pituitaries at different stages of the estrous cycle. *Endocrinology* **136**, 62-68.
- Funston RN, Jr. GES, Klindt J and Roberts AJ. (1996). Insulin-like growth factor-I and insulin-like growth factor-binding proteins in bovine serum and follicular fluid before and after the preovulatory surge of luteinizing hormone. *Biol. Reprod.* **55**, 1390-1396.
- Gadsby JE, Lovdal JA, Samaras S, Barber JS and Hammond JM. (1996). Expression of the messenger ribonucleic acids for insulin-like growth factor-I and insulin-like growth factor binding proteins in porcine corpora lutea. *Biol. Reprod.* **54**, 339-346.
- Garrett WM and Guthrie HD. (1996). Expression of androgen receptors and steroidogenic enzymes in relation to follicular growth and atresia following ovulation in pigs. *Biol. Reprod.* **55**, 949-955.
- Getzenberg RH, Pienta KJ and Coffey DS. (1990). The tissue matrix: Cell dynamics and hormone action. *Endocr. Rev* **11**, 399-417.
- Giudice LC. (1992). Insulin-like growth factors and ovarian follicular development. *Endocr. Rev.* **13**, 641-669.
- Goddard C, Wilkie RS and Dunn IC. (1988). The relationship between insulin-like growth factor-I, growth hormone, thyroid hormones and insulin in chickens selected for growth. *Dom. Anim. Endocrinol.* **5**, 165-176.
- Gong JG, Bramley TA and Webb R. (1991). The effect of recombinant bovine somatotropin on ovarian function in heifers: Follicular populations and peripheral hormones. *Biol. Reprod.* **45**, 941-949.
- Gong JG, Bramley TA and Webb R. (1993a). The effect of recombinant bovine somatotrophin on ovarian follicular growth and development in heifers. *J. Reprod. Fertil.* **97**, 247-254.

- Gong JG, Bramley T, Wilmut I and Webb R. (1993b). Effect of recombinant bovine somatotropin on superovulatory response to pregnant mare serum gonadotropin in heifers. *Biol. Reprod.* **48**, 1141-1149.
- Gong JG, McBride D, Bramley TA and Webb R. (1993c). Effects of recombinant bovine somatotrophin, insulin-like growth factor-1 and insulin on the proliferation of bovine granulosa cells in-vivo. *J. Endocrinol.* **139**, 67-75.
- Gong JG, McBride D, Bramley TA and Webb R. (1994). Effects of recombinant bovine somatotrophin, insulin-like growth factor-I and insulin on bovine granulosa cell steroidogenesis in vitro. *J. Endocrinol.* **143**, 157-164.
- Gong JG, Bramley TA, Gutierrez CG, Peters AR and Webb R. (1995). Effects of chronic treatment with a gonadotrophin-releasing hormone agonist on peripheral concentrations of FSH and LH, and ovulation function in heifers. *J. Reprod. Fertil.* **105**, 263-270.
- Gong JG, Campbell BK, Bramley TA, Gutierrez CG, Peters AR and Webb R. (1996a). Suppression in the secretion of follicle-stimulating hormone and luteinizing hormone, and ovarian follicle development in heifers continuously infused with a gonadotropin-releasing hormone agonist. *Biol. Reprod.* **55**, 68-74.
- Gong JG, Campbell BK, Bramley TA and Webb R. (1996b). Treatment with recombinant bovine somatotrophin enhances ovarian follicle development and increases the secretion of insulin-like growth factor-I by ovarian follicle in ewes. *Anim. Reprod. Sci.* **41**, 13-26.
- Gong JG, Campbell BK and Webb R. (1997). Suppression of FSH secretion by treatment with oestradiol and bovine follicular fluid in heifers: An effect independent of GnRH action? In *UK National Ovarian Workshop*, Bristol. UK.
- Gonzalez-Padilla E, Wiltban JN and Niswender GD. (1975). Puberty in beef heifers. 1. The interrelationship between pituitary, hypothalamic and ovarian hormones. *J. Anim. Sci.* **40**, 1091-1104.
- Gosden RG, Hunter RHF, Telfer E, Torrance C and Brown N. (1988). Physiological factors underlying the formation of ovarian follicular fluid. *J. Reprod. Fertil.* **82**, 813-825.
- Gosden RG, Baird DT, Wade JCW and Webb R. (1994). Restoration of fertility to oophorectomized sheep by ovarian autografts stored at -196C. *Human Reprod.* **9**, 597-603.
- Gougeon A. (1996). Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocr. Rev.* **17**, 121-155.
- Grimard B, Humblot P, Ponter AA, Mialot JP, Sauvart D and Thibier M. (1995). Influence of postpartum energy restriction on energy status, plasma LH and oestradiol secretion and follicular development in suckled beef cows. *J. Reprod. Fertil.* **104**, 173-179.
- Grimes RW, Matton P and Ireland JJ. (1987). A comparison of histological and non-histological indices of atresia and follicular function. *Biol. Reprod.* **37**, 82-88.

- Guilbault LA, Grasso F, Lussier JG, Rouillier P and Matton P. (1991). Decreased superovulatory responses in heifers superovulated in the presence of a dominant follicle. *J. Reprod. Fertil.* **91**, 81-89.
- Gutierrez CG. (1992). Comparacion de la foliculogenesis y ciclos estrales de novillonas cebu y cebu-holstein durante los meses de marzo a junio en el tropico humedo de Mexico. MSc thesis, Universidad Nacional Autonoma de Mexico.
- Gutierrez CG, Galina CS, Zarco L and Rubio I. (1994). Pattern of follicular growth during prepuberal anoestrous and transition from anoestrous to oestrous cycles in *Bos indicus* heifers. *Adv. Agri. Res.* **3**, 1-11.
- Hakuno N, Koji T, Yano T, Kobayashi N, Tsutsumi O, Taketani Y and Nakane P. (1996). Fas/APO-1/CD95 system as a mediator of granulosa cell apoptosis in ovarian follicle atresia. *Endocrinology* **137**, 1938-1948.
- Hammond J. (1927). The physiology of reproduction in the cow, pp. 5-8. Cambridge University Press.
- Hammond JM, Mondschein JS, Samaras SE, Smith SA and Hagen DR. (1991). The ovarian insulin-like growth factor system. *J. Reprod. Fertil. Suppl.* **43**, 199-208.
- Hammond JM, Samaras SE, Grimes R, Leighton J, Barber J, Canning SF and Guthrie HD. (1993). The role of insulin-like growth factors and epidermal growth factor-related peptides in intraovarian regulation in the pig ovary. *J. Reprod. Fertil. Suppl.* **48**, 117-125.
- Hansel W and Convey E. (1983). Physiology of the oestrous cycle. *J. Anim. Sci.* **57**, 404-424.
- Hayden JM, Marten NW, Burke EJ and Straus DS. (1994). The effect of fasting on insulin-like growth factor-I nuclear transcript abundance in rat liver. *Endocrinology* **134**, 760-768.
- Heller DT, Cahil DM and Schultz RM. (1981). Biochemical studies of mammalian oogenesis: Metabolic cooperativity between granulosa cells and growing mouse oocytes. *Dev. Biol.* **84**, 455-464.
- Heralds RL and Schultz RM. (1984). Regulation of mouse oocyte growth: probable nutritional role for intercellular communication between follicle cells and oocytes in oocyte growth. *J. Exp. Zool.* **229**, 317-325.
- Hernandez ER. (1995). Regulation of the genes for insulin-like growth factor (IGF) I and II and their receptors by steroids and gonadotropins in the ovary. *J. Steroid. Biochem. Molec. Biol.* **53**, 219-221.
- Herrier A, Einspanier R, Schams D and Nieman H. (1994). Effect of recombinant bovine somatotropin (rBST) on follicular IGF-1 contents and the ovarian response following superovulatory treatment in dairy cows; a preliminary study. *Theriogenology* **41**, 600-611.
- Hileman SM, Schillo KK, Kearman JM, Hall JB and Mohapatra S. (1991). Effects of metabolic fuel restriction on patterns of LH and GH in ovariectomized lambs. *Biol. Reprod. (Suppl 1)*, 138.

- Hileman SM, Schillo KK and Hall JB. (1993). Effects of acute, intracerebroventricular administration of insulin on serum concentrations of luteinizing hormone, insulin, and glucose in ovariectomized lambs during restricted and *Ad libitum* feed intake. *Biol. Reprod.* **48**, 117-124.
- Hillier SG, Whitelaw PF and Smyth CD. (1994). Follicular oestrogen synthesis: the 'two-cell, two-gonadotrophin' model revisited. *Mol. Cell. Endocrinol.* **100**, 51-54.
- Hinshelwood MM, Kamel F, Dierschke DJ and Hauser ER. (1991). Effects of charcoal-extracted follicular fluid on reproductive function in postpartum cows. *Dom. Anim. Endocrinol.* **8**, 37-54.
- Hintz RL and Liu F. (1977). Demonstration of specific plasma protein binding sites for somatomedin. *J. Clin. Endocrinol. Metabol.* **45**, 988-995.
- Hirshfield AN, Waldemar A and Schmidt WA. (1987). Kinetic aspects of follicular development in the rat. *Adv. Exp. Med. Biol.* **219**, 737-742.
- Hirshfield AN. (1989). Rescue of atretic follicles in vitro and in vivo. *Biol. Reprod.* **40**, 181-190.
- Hirshfield AN. (1991). Development of follicles in the mammalian ovary. *Int. Rev. Cytol.* **124**, 43-101.
- Hockenbery D. (1995). Defining apoptosis. *Am. J. Pathology* **146**, 16-19.
- Hodgkinson S, Bass JJ and Gluckman PD. (1991). Plasma IGF-1 binding proteins in sheep: Effect of recombinant growth hormone treatment and nutritional status. *Dom. Anim. Endocrinol.* **8**, 343-351.
- Holly JMP and Hughes SCC. (1994). Measuring insulin-like growth factors: why and how? *J. Endocrinol.* **140**, 165-169.
- Homburg R, West C, Torresani T and Jacobs HS. (1990). Cotreatment with human growth hormone and gonadotropins for induction of ovulation: a controlled clinical trial. *Fertil. Steril.* **53**, 254-260.
- Hossenlopp P, Seurin D, Segovia-Quinson B, Hardouin S and Binoux M. (1986). Analysis of serum insulin-like growth factor binding proteins using western blotting: use of the method for titration of the binding proteins and competitive binding studies. *Analyt. Biochem.* **154**, 138-143.
- Hossner KL, McCusker RH and Dodson MV. (1997). Insulin-like growth factors and their binding proteins in domestic animals. *Anim. Sci.* **1997**, 1-15.
- Howard HJ and Ford JJ. (1994). Differential steroidogenic response of subpopulations of porcine granulosa cells to insulin-like growth factor-I (IGF-I) or IGF-I analogs. *Biol. Reprod.* **51**, 108-115.
- Hsu SY, Lai RJM, Finegold M and Hsueh AJW. (1996). Targeted overexpression of Bcl-2 in ovaries of transgenic mice leads to decreased follicle apoptosis, enhanced folliculogenesis, and increased germ cell tumorigenesis. *Endocrinology* **137**, 4837-4843.

- Hsueh AJW, Billig H and Tsafiriri A. (1994). Ovarian follicle atresia: A hormonally controlled apoptotic process. *Endocr. Rev.* **15**, 707-724.
- Hua KM, Hodgkinson SC and Bass JJ. (1995). Differential regulation of plasma levels of insulin-like growth factors-I and -II by nutrition, age and growth hormone treatment in sheep. *J. Endocrinol.* **147**, 507-516.
- Huang H, Rajkumar K and Murphy LJ. (1997). Reduced fecundity in insulin-like growth factor-binding protein-1 transgenic mice. *Biol. Reprod.* **56**, 284-289.
- Hughes FM and Gorospe WC. (1991). Biochemical identification of apoptosis (programmed cell death) in granulosa cells: evidence for a potential mechanism underlying follicular atresia. *Endocrinology* **129**, 2415-2422.
- I'Anson H, Quint EH, Wood RI, England BG and Foster DL. (1994). Adrenal axis and hypogonadotropism in growth-restricted female lamb. *Biol. Reprod.* **50**, 137-143.
- Imakawa K, Day ML, Garcia-Winder M, Zalesky DD, Kittok RJ, Schanbacher BD and Kinder JE. (1986a). Endocrine changes during restoration of estrous cycles following induction of anestrus by restricted nutrient intake in beef heifers. *J. Anim. Sci.* **63**, 565-571.
- Imakawa K, Day ML, Zalesky DD, Garcia-Winder M, Kittok RJ and Kinder JE. (1986b). Influence of dietary-induced weight changes on serum luteinizing hormone, estrogen and progesterone in the bovine female. *Biol. Reprod.* **35**, 377-384.
- Ireland JJ and Roche JF. (1983). Development of nonovulatory antral follicles in heifers: Changes in steroids in follicular fluid and receptors for gonadotropins. *Endocrinology* **112**, 150-156.
- Jacobs HS. (1993). Evidence for the utility of growth hormone in the enhancement of ovulation. In *The somatotrophic axis and the reproductive process in health and disease*, pp. 28. Serono Symposia, Baltimore, Maryland.
- Jolly PD, Tisdall DJ, Heath DA, Lun S and McNatty KP. (1994). Apoptosis in bovine granulosa cells in relation to steroid synthesis, cyclic adenosine 3',5'-monophosphate response to follicle-stimulating hormone and luteinizing hormone, and follicular atresia. *Biol. Reprod.* **51**, 934-944.
- Jolly PD, McDougall S, Fitzpatrick LA, Macmillan KL and Entwistle KW. (1995). Physiological effects of undernutrition on postpartum anoestrus in cows. *J. Reprod. Fertil.* **49**, 477-492.
- Jones JJ and Clemmons DR. (1995). Insulin-like growth factors and their binding proteins: Biological actions. *Endocr. Rev.* **16**, 3-34.
- Juliano R. (1996). Cooperation between soluble factors and integrin-mediated cell anchorage in the control of cell growth and differentiation. *Trends Endocrinol. Metabol.* **18**, 911-917.
- Kahn CR, Baird KL, Flier JS, Grunfeld C, Harmon JT, Harrison LC, Karlsson FA, Kasuga M, King GL, Lang UC, Podskalny JM and Obberghen EV. (1981). Insulin

receptors, receptor antibodies, and the mechanisms of insulin action. *Rec. Prog. Horm. Res.* **37**, 477-538.

Kaipia A, Chun S, Eisenhauer K and Hsueh AJW. (1996). Tumor necrosis factor- α and its second messenger, ceramide, stimulate apoptosis in culture ovarian follicles. *Endocrinology* **137**, 4884-4870.

Kanzaki M, Hattori MA, Horiuchi R and Kojima I. (1994). Co-ordinate actions of FSH and insulin-like growth factor-I on LH receptor expression in rat granulosa cells. *J. Endocrinol.* **141**, 301-308.

Kaur H and Arora SP. (1995). Dietary effects on ruminant livestock reproduction with particular reference to protein. *Nutrit. Res. Rev.* **8**, 121-136.

Kawate N, Inaba T and Mori J. (1993). Effects of cortisol on the amounts of estradiol-17 β and progesterone secreted and the number of luteinizing hormone receptors in cultured bovine granulosa cells. *Anim. Reprod. Sci.* **32**, 15-25.

Kazmer GW, Barnes MA, Akers RM and Pearson RE. (1986). Effect of genetic selection for milk yield and increased milking frequency on plasma growth hormone and prolactin concentration in holstein cows. *J. Anim. Sci.* **63**, 1220-1227.

Kerr JFR, Willie AH and Currie AR. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26**, 239-257.

Kerr JFR, Winterford CM and Harmon BV. (1994). Morphological criteria for identifying apoptosis. In *Cell biology: A laboratory handbook*, vol. 1 (ed. Celis), pp. 319-329. Academic Press, Inc., San Diego, Cal.

Kesner JS, Convey EM and Anderson CR. (1981). Evidence that estradiol induces the preovulatory LH surge in cattle by increasing pituitary sensitivity to LHRH and the increasing LHRH release. *Endocrinology* **108**, 1386.

Kirby CJ, Thatcher WW, Collier RJ, Simmen FA and Lucy MC. (1996). Effects of growth hormone and pregnancy on expression of growth hormone receptor, insulin-like growth factor binding protein-2 and -3 genes in bovine uterus, ovary, and oviduct. *Biol. Reprod.* **55**, 996-1002.

Kirby CJ, Smith MF, Keisler DH and Lucy MC. (1997). Follicular function in lactating dairy cows treated with sustained-release bovine somatotropin. *J. Dairy Sci.* **80**, 273-285.

Kitson J, Raven T, Jiang YP, Goeddel DV, Giles KM, Pun KT, Grinham CJ, Brown R and Farrow SN. (1996). A death-domain-containing receptor that mediates apoptosis. *Nature* **384**, 372-375.

Ko JCH, Kastelic JP, Campo MRD and Ginther OJ. (1991). Effects of a dominant follicle on ovarian follicular dynamics during the oestrous cycle in heifers. *J. Reprod. Fertil.* **91**, 511-519.

Lamond DR. (1970). The influence of undernutrition on reproduction of the cow. *Anim. Breed. Abstr.* **38**, 359-372.

- Land, RB (1970). Number of oocytes present at birth in the ovaries of pure and Finish Landrace cross Blackface and Welsh sheep. *J. Reprod. Fertil.* **21**, 517-521.
- Landefeld TD, Ebling FJP, Suttie JM, Vannerson LA, Padmanabhan V, Beitins IZ and Foster DL. (1989). Metabolic interfaces between growth and reproduction. II. Characterization of changes in messenger ribonucleic acid concentrations of gonadotropin subunits, growth hormone, and prolactin in nutritionally growth-limited lambs and the differential effects of increased nutrition. *Endocrinology* **125**, 351-356.
- Langhout DJ, Spicer LJ and Geisert RD. (1991). Development of a culture system for bovine granulosa cells: effects of growth hormone, estradiol, and gonadotropins on cell proliferation, steroidogenesis, and protein synthesis. *J. Anim. Sci.* **69**, 3321-3334.
- Larson GH, Mallory DS, Dailey RA and Lewis PE. (1991). Gonadotropin concentrations follicular development and luteal function in pituitary stalk-transected ewes treated with some bovine follicular fluids. *J. Anim. Sci.* **69**, 4101-4111.
- Lavranos TC, Rodgers HF, Bertonecello I and Rodgers RJ. (1994). Anchorage-independent culture of bovine granulosa cells: the effect of basic fibroblast growth factor and dibutyryl cAMP on cell division and differentiation. *Exp. Cell Res.* **211**, 245-251.
- Law AS, Baxter G, Logue DN, O'Shea T and Webb R. (1992). Evidence for the action of bovine follicular fluid factor(s) other than inhibin in suppressing follicular development and delaying the oestrus in heifers. *J. Reprod. Fertil.* **96**, 603-616.
- Lee CY and Henricks DM. (1990). Comparisons of various acidic treatments of bovine serum on insulin-like growth factor-I immunoreactivity and binding activity. *J. Endocrinol.* **127**, 139-148.
- Leeuwenberg BR, Hurst PR and McNatty KP. (1995). Expression of IGF-I mRNA in the ovine ovary. *J. Mol. Endocrinol.* **15**, 251-258.
- Leeuwenberg BR, Hudson NL, Moore LG, Hurst PR and McNatty KP. (1996). Peripheral and ovarian IGF-I concentrations during the ovine oestrous cycle. *J. Endocrinol.* **148**, 281-289.
- Li R, Phillips DM and Mather JP. (1995). Activin promotes ovarian follicle development in vitro. *Endocrinology* **136**, 849-856.
- Lobb DK and Dorrington J. (1992). Intraovarian regulation of follicular development. *Anim. Reprod. Sci.* **28**, 343-354.
- Logan A and Hill DJ. (1992). Bioavailability: Is this a key event in regulating the actions of peptide growth factors? *J. Endocrinol.* **134**, 157-161.
- Logothetopoulos J, Dorrington J, Bailey D and Stratis M. (1995). Dynamics of follicular growth and atresia of large follicles during the ovarian cycle of guinea pig: fate of the degenerating follicles a quantitative study. *Anat. Rec.* **243**, 37-48.
- Lovendahl P, Angus KD and Woolliams JA. (1991). The effect of genetic selection for milk yield on the response to growth hormone secretagogues in immature cattle. *J. Endocrinol.* **128**, 419-424.

- Luck MR, Rodgers RJ and Findlay JK. (1990). Secretion and gene expression of inhibin, oxytocin and steroid hormones during the in vitro differentiation of bovine granulosa cells. *Reprod. Fertil. Dev.* **2**, 11-25.
- Luck M. (1994). The gonadal extracellular matrix. *Oxford Rev. Reprod. Biol.* **16**, 34-85.
- Lucy MC, Staples CR, Michel FM and Thatcher WW. (1991a). Energy Balance and size and number of ovarian follicles detected by ultrasonography in early postpartum dairy cows. *J. Dairy Sci.* **74**, 473-482.
- Lucy MC, Staples CR, Michel FM and Thatcher WW. (1991b). Effect of feeding calcium soaps to early postpartum dairy cows on plasma prostaglandin F_{2α}, luteinizing hormone, and follicular growth. *J. Dairy Sci.* **74**, 483-489.
- Lucy MC, Beck J, Staples CR, Head HH, DelaSota RL and Thatcher WW. (1992). Follicular dynamics, plasma metabolites, hormones and insulin-like growth factor (IGF-1) in lactating cows with positive or negative energy balance during the preovulatory period. *Reprod. Nutr. Dev.* **32**, 331-341.
- Lucy MC, Collier RJ, Kitchell ML, Dibner JJ, Hauser SD and Krivi GG. (1993a). Immunohistochemical and nucleic acid analysis of somatotropin receptor populations in the bovine ovary. *Biol. Reprod.* **48**, 1219-1227.
- Lucy MC, DelaSota RL, Staples CR and Thatcher WW. (1993b). Ovarian follicular populations in lactating dairy cows treated with recombinant bovine somatotropin (Sometribove) or saline and fed diets differing in fat content and energy. *J. Dairy Sci.* **76**, 1014-1027.
- Lund PK. (1994). Insulin-like growth factor I: Molecular Biology and relevance to tissue-specific expression and action. *Rec. Prog. Horm. Res.* **49**, 125-148.
- Lussier JG, Matton P and Dufour JJ. (1987). Growth rates of follicles in the ovary of the cow. *J. Reprod. Fertil.* **81**, 301-307.
- Magarelli PC, Zachow RJ and Magoffin DA. (1996). Development and hormonal regulation of rat theca-cell differentiation factor secretion in ovarian follicles. *Biol. Reprod.* **55**, 416-420.
- Majno G and Joris I. (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. *Am. J. Pathology* **146**, 3-15.
- Mariana J. (1980). Some remarks on the long term trends and some short regulations in the follicular growth. In *IX International Congress of Animal Reproduction*, Spain.
- Martin SJ, Green DR and Cotter TG. (1994). Dicing with death: dissecting the components of the apoptosis machinery. *Trends Biochem. Sci.* **19**, 26-30.
- Matamoros IA, Cox NM and Moore AB. (1990). Exogenous insulin and additional energy affect follicular distribution, follicular steroid concentrations, and granulosa cell human chorionic gonadotropin binding in swine. *Biol. Reprod.* **43**, 1-7.

- Matamoros IA, Cox NM and Moore AB. (1991). Effects of exogenous insulin and body condition on metabolic hormones and gonadotropin-induced follicular development in prepuberal gilts. *J. Anim. Sci.* **69**, 2081-2091.
- Matton P, Adelakoun V, Couture Y and Dufour JJ. (1981). Growth and replacement of the bovine ovarian follicles during the estrous cycle. *J. Anim. Sci.* **52**, 813-820.
- Maurasse C, Marton P and Dufour JJ. (1985). Ovarian follicular populations at two stages of an estrous cycle in heifers given high energy diets. *J. Anim. Sci.* **61**, 1194-1200.
- Maxwell SE and Delaney HD. (1990). *Designing experiment and analyzing data*. Brooks/Cole Publishing Co., California.
- McCann JP and Hansel W. (1986). Relationships between insulin and glucose metabolism in pituitary-ovarian functions in fasted heifers. *Biol. Reprod.* **34**, 630-641.
- McClellan MC, Diekman MA, Abel JH Jr. and Niswender GD. (1975). Luteinizing hormone, progesterone and the morphological development of normal and superovulated corpora lutea in sheep. *Cell Tiss. Res.* **164**, 291-307.
- McClure TJ. (1968). Hypoglycaemia, an apparent cause of infertility of lactating cows. *Br. vet. J.* **124**, 126-130.
- McClure TJ, Nancarrow CD and Radford HM. (1978). The effect of 2-Deoxy-D-glucose on ovarian function of cattle. *Aust. J. Biol. Sci.* **31**, 183-186.
- McGuire MA, Vicini JL, Bauman DE and Veenhuizen JJ. (1992). Insulin-like growth factors and binding proteins in ruminants and their nutritional regulation. *J. Anim. Sci.* **70**, 2901-2910.
- McLaren A. (1980). Folliculogenesis and ova production. In *IX International Congress of Animal Reproduction*, Spain.
- McNeilly AS, Picton HM, Campbell BK and Baird DT. (1991). Gonadotrophic control of follicle growth in the ewe. *J. Reprod. Fert. Suppl.* **43**, 177-186.
- Meikrantz W and Schlegel R. (1995). Apoptosis and the cell-cycle. *J. Cell Biochem.* **58**, 160-174.
- Merchant LH. (1991). Gametogenesis. In *Temas selectos de biología de la reproducción* (ed. Dominguez), pp. 11-40. MA Porrúa, Mexico.
- Meurer KA, Cox NM, Matamoros IA and Tubbs RC. (1991). Decreased follicular steroids and insulin-like growth factor-1 and increased atresia in diabetic gilts during follicular growth stimulated with PMSG. *J. Reprod. Fert.* **91**, 187-196.
- Monget P, Monniaux D, Pisselet C and Durand P. (1993). Changes in insulin-like growth factor-1 (IGF-1), IGF-II, and their binding proteins during growth and atresia of ovine ovarian follicles. *Endocrinology* **132**, 1438-1446.
- Monget P and Monniaux D. (1995). Growth factors and the control of folliculogenesis. *J. Reprod. Fert. Suppl.* **49**, 321-333.

- Moore GPM and Lintern-Moore S. (1979). Patterns of gene activity during ovum formation in the mouse. *Ann. Biol. Anim. Bioch. Biophys.* **19**, 1409-1417.
- Mounzih K, Lu R and Chehab FF. (1997). Leptin treatment rescues the sterility of genetically obese ob/ob males. *Endocrinology* **138**, 1190-1193.
- Mulheron CW, Quattropani SL and Nolin JM. (1987). On the intrinsic control of the developmental transition from primordial to primary follicle. *Adv. Exp. Med. Biol.* **219**, 737-742.
- Murao K, Sato M, Mizobuchi M, Niimi M, Ishida T and Takahara JJ. (1994). Acute effects of hypoglycemia and hyperglycemia on hypothalamic growth hormone-releasing hormone and somatostatin gene expression in the rat. *Endocrinology* **134**, 418-423.
- Murphy MG, Enright WJ, Crowe MA, McConnell K, Spicer LJ, Boland MP and Roche JF. (1991). Effect of dietary intake on pattern of growth of dominant follicles during the oestrous cycle in beef heifers. *J. Reprod. Fertil.* **92**, 333-338.
- Nayudu PL and Osborn SM. (1992). Factors influencing the rate of preantral and antral growth of mouse ovarian follicles in vitro. *J. Reprod. Fertil.* **1992**, 349-362.
- Nishimori K and Matzuk M. (1996). Transgenic mice in the analysis of reproductive development and function. *Rev. Reprod.* **1**, 203-212.
- O'Shea JD, Hay MF and Cran DG. (1978). Ultrastructural changes in the theca interna during follicular atresia in sheep. *J. Reprod. Fertil.* **54**, 183-187.
- Ober JA and Malven PV. (1992). Effect of growth retardation on pituitary hormone and hypothalamic neuropeptide Y in ovariectomized sheep. *Neuroendocrinol.* **56**, 331-339.
- Oh YM, Nagalla SR, Yamanaka Y, Kim HS, Wilson E and Rosenfeld RG. (1996). Synthesis and characterization of insulin-like growth factor-binding protein (IGFBP)-7 - recombinant human mac25 protein specifically binds IGF-I and IGF-II. *J. Biol. Chemistry* **271**, 30322-30325.
- Ojeda SR and Dissen GA. (1994). Developmental regulation of the ovary via growth factor tyrosine kinase receptor. *Trend. Endocrinol. Metabol.* **8**, 317-323.
- Oliver JE, Aitman TJ, Powell JF, Wilson CA and Clayton RN. (1989). Insulin-like growth factor I gene expression in the rat ovary is confined to the granulosa cells of developing follicles. *Endocrinology* **124**, 2671-2679.
- Osmond TJ, Carr WR, Hinks CJM, Land RB and Hill WG. (1981). Physiological attributes as possible selection criteria for milk production 2. plasma insulin, triiodothyronine and thyroxine in bulls. *Anim. Prod.* **32**, 159-163.
- Owens PC, Johnson RJ, Campbell RG and Ballard GJ. (1990). Growth hormone increases insulin-like growth factor -i (IGF-I) and decreases IGF-II in plasma of growing pigs. *J. Endocrinol.* **124**, 269-275.
- Pao CI, Farmer PK, Begovic S, Villafuerte BC, Wu G, Robertson DG and Phillips LS. (1993). Regulation of insulin-like growth factor-1 (IGF-1) and IGF-binding

protein 1 gene transcription by hormones and provision of amino acids in rat hepatocytes. *Mol. Endocrinol.* **7**, 1561-1568.

Peel CJ and Bauman DE. (1987). Somatotropin and lactation. *J. Dairy Sci.* **70**, 474-486.

Pell JM and Bates PC. (1990). The nutritional regulation of growth hormone action. *Nutrit. Res. Rev.* **3**, 163-192.

Peluso JJ, Luciano AM, Pappalardo A and White BA. (1995). Cellular and molecular mechanisms that mediate insulin-dependent rat granulosa cell mitosis. *Biol. Reprod.* **52**, 124-130.

Perks CM, Denning-Kendall PA, Gilmour RS and Wathes DC. (1995). Localization of messenger ribonucleic acids for insulin-like growth factor I (IGF-I), IGF-II, and the type I IGF receptor in the ovine ovary throughout the estrous cycle. *Endocrinology* **136**, 5266-5273.

Perotti L, Greppi GF, Rizzi N, Serrantoni M, Corti M and Scotti GG. (1989). Andamento di alcuni metaboliti plasmatici e dell'insulina in bovine Frisone ad alta e bassa produzione nel corso della lattazione. *Zoot. Nutr. Anim.* **15**, 381-388.

Picton HM, Tsonis CG and McNeilly AS. (1990). FSH causes a time-dependent stimulation of preovulatory follicle growth in the absence of pulsatile LH secretion in ewes chronically treated with gonadotrophin-releasing hormone agonist. *J. Endocrinol.* **126**, 297-307.

Picton HM, Campbell BK and Hunter MG. (1994). Maintenance of aromatase activity in porcine granulosa cells in serum free culture. *J. Reprod. Fertil. Abstr. Series* **14**, abstr. 1.

Pierson RA and Ginther OJ. (1988). Ultrasonic imaging of the ovaries and uterus in cattle. *Theriogenology* **28**, 94-100.

Poretsky L and Kalin MF. (1987). The gonadotropic function of insulin. *Endocr. Rev.* **8**, 132-141.

Poretsky L. (1991). On the paradox of insulin-induced hyperandrogenism in insulin-resistant states. *Endocr. Rev.* **12**, 3-13.

Price CA and Webb R. (1989). Ovarian response to hCG treatment during the oestrous cycle in heifers. *J. Reprod. Fertil.* **86**, 303-308.

Putu IG, Lubis A and Flechter IC. (1986). Reproduction in swamp buffalo cows after oestrous synchronization at two mating seasons and two levels of nutrition. *Anim. Reprod. Sci.* **11**, 99-109.

Rajakoski E. (1960). The ovarian follicular system in sexually mature heifers with special reference to seasonal, cyclical and left-right variations. *Acta Endocrinol. Suppl.* **52**, 1-68.

Ralph JH. (1996). Factors affecting follicle and oocyte development in cattle. Ph.D. thesis, University of Edinburgh.

- Redmer DA, Dai Y, Charnock-Jones DS, Smith SK, Reynolds LP and Moor RM (1996) Characterization and expression of vascular endothelial growth factor (VEGF) in the ovine corpus luteum. *J. Reprod. Fertil.* **108**, 157-165.
- Reynolds ES. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell. Biol.* **17**, 203-212.
- Rhind SM, Bramley TA, Wright IA, and McMillen S. (1992). FSH and LH receptor concentrations in large ovarian follicles of beef cows in high and low levels of body condition at nine weeks postpartum. *Reprod. Fertil. Develop.* **4**, 515-522.
- Effect of body condition score of ewes in the secretion of LH and FSH and the pituitary response to gonadotrophin-releasing hormone. *J. Endocrinol.* **120**, 497-502.
- Rhind SM, McMillen S, McKelvey WAC, Rodriguez-Herrejon FF and McNeilly AS. (1989). Effect of body condition score of ewes in the secretion of LH and FSH and the pituitary response to gonadotrophin-releasing hormone. *J. Endocrinol.* **120**, 497-502.
- Rhodes FM, Fitzpatrick LA, Entwistle KW and De'ath G. (1995). Sequential changes in ovarian follicular dynamics in *Bos indicus* heifers before and after nutritional anoestrus. *J. Reprod. Fertil.* **104**, 41-49.
- Richards MW, Wettemann RP and Schoenemann HM. (1989a). Nutritional anestrus in beef cows: body weight change, body condition, luteinizing hormone in serum and ovarian activity. *J. Anim. Sci.* **67**, 1520-1526.
- Richards MW, Wettemann RP and Schoenemann HM. (1989b). Nutritional anestrus in beef cows: Concentrations of glucose and nonesterified fatty acids in plasma and insulin serum. *J. Anim. Sci.* **67**, 2354-2362.
- Richards MW, Spicer LJ and Wettemann RP. (1995). Influence of diet and ambient temperature on bovine serum insulin-like growth factor-I and thyroxine: relationships with non-esterified fatty acids, glucose, insulin, luteinizing hormone and progesterone. *Anim. Prod. Sci.* **37**, 267-679.
- Rivero F, Goya L and Pascual-Leone AM. (1994). Comparison of extraction methods for insulin-like growth factor-binding proteins prior to measurement of insulin-like growth factor I in undernourished neonatal and adult rat serum. *J. Endocrinol.* **140**, 257-263.
- Roberson MS, Stumpf TT, Wolfe MW, Kittok RJ and Kinder JE. (1991). Influence of direction of body weight change on pattern of gonadotropin secretion in ovariectomized beef heifers of equivalent body weight. *J. Anim. Sci.* **69**, 1616-1625.
- Roberson MS, Stumpf TT, Wolfe MW, Cupp AS, Kojima N, Werth LA, Kittok RJ and Kinder JE. (1992). Circulating gonadotrophins during a period of restricted energy intake in relation to body condition in heifers. *J. Reprod. Fertil.* **96**, 461-469.
- Roberts AJ and Echterkamp SE. (1994). In vitro production of estradiol by bovine granulosa cells: evaluation of culture condition, stage of follicular development, and location of cells within follicles. *Biol. Reprod.* **51**, 273-282.

- Robinson JJ. (1990). Nutrition in the reproduction of farm animals. *Nutrit. Res. Rev.* **3**, 253-276.
- Robinson JJ. (1996). Nutrition and reproduction. *Anim. Reprod. Sci.* **42**, 25-34.
- Roche JE and Boland MP. (1991). Turnover of dominant follicles in cattle of different reproductive states. *Theriogenology* **35**, 81-90.
- Rodgers RJ, Vella CA, Rodgers HF, Scott K and Lavranos TC. (1996). Production of extracellular matrix, fibronectin and steroidogenic enzymes, and growth of bovine granulosa cells in anchorage-independent culture. *Reprod. Fertil. Dev.* **8**, 249-257.
- Rodgers BD. (1996). Catabolic hormones and growth hormone resistance in acquired immunodeficiency syndrome and other catabolic states. *Proc Soc. Exp. Biol. Med.* **212**, 324-331.
- Roeder RA, Garber MJ, Dalke BS, Kasser TR, Veenhuizen J and Schelling GT. (1994). Effects of recombinant bovine somatotropin implants on serum concentrations of somatotropin, insulin-like growth factor-I and blood urea nitrogen in steers. *Growth Regulation* **4**, 101-107.
- Ropke R, Schams D, Schwarz FJ and Kirchgessner M. (1994). Growth-related hormones in plasma of bulls, steers and heifers given food with two different energy levels. *Anim. Prod.* **59**, 367-377.
- Rosenfeld RG, Oh Y, Hwa V, Kim HS, Nagalla S, Roberts C, Vorwek P, Wanek D, Wilson E, Yamanaka Y and Yang DH. (1997). Insulin-like growth factor binding proteins: new perspectives on their roles as regulators of growth. *J. Endocrinol. Suppl.* **152**, S3.
- Rouillier P, Matton P, Sirard MA and Guilbault LA. (1996). Follicle-stimulating hormone-induced estradiol and progesterone production by bovine antral and mural granulosa cells cultured in vitro in a completely defined medium. *J. Anim. Sci.* **74**, 3012-3019.
- Rusbridge SM, Bramley TA and Webb R. (1993). A comparison of progesterone production by bovine luteal cells from GnRH-induced corpora lutea (CL) and spontaneously-formed CL *in vitro*. *J. Reprod. Fertil. Abstr. Series* **12**, Abstract 21.
- Samaras SE, Guthrie HD, Barber JA and Hammond JM. (1993). Expression of the mRNAs for the insulin-like growth factors and their binding proteins during development of porcine ovarian follicles. *Endocrinology* **133**, 2395-2398.
- Samaras SE, Hagen DR, Bryan KA, Mondschein JS, Canning SF and Hammond JM. (1994). Effects of growth hormone and gonadotropin on the insulin-like growth factor system in the porcine ovary. *Biol. Reprod.* **50**, 178-186.
- Samaras SE, Canning SF, Barber JA, Simmen FA and Hammond JM. (1996). Regulation of insulin-like growth factor I biosynthesis in porcine granulosa cells. *Endocrinology* **137**, 4657-4664.
- Saumande J. (1991). Culture of bovine granulosa cells in a chemically defined serum-free medium: the effect of insulin and fibronectin on the response to FSH. *J. Steroid Biochem. Mol. Biol.* **38**, 189-196.

- Savio JD, Boland MP, Hynes N and Roche JF. (1990). Resumption of follicular activity in the early post-partum period of dairy cows. *J. Reprod. Fertil.* **88**, 569-579.
- Savio JD, Thatcher WW, Badinga L, Sota RLdl and Wolfenson D. (1993). Regulation of dominant follicle turnover during the oestrous cycle of cows. *J. Reprod. Fertil.* **97**, 197-203.
- Schmitt EJP, Barros CM, Fields PA, Fields MJ, Diaz T, Kluge JM and Thatcher WW. (1996a). A cellular and endocrine characterization of the original and induced corpus luteum after administration of a gonadotrophin-releasing hormone agonist or human chorionic gonadotropin on day five of the oestrous cycle. *J. Anim. Sci.* **74**, 1915-1929.
- Schmitt EJP, Diaz T, Barros CM, Sota RLdl, Drost M, Fredriksson EW, Staples CR, Thorner R and Thatcher WW. (1996b). Differential response of the luteal phase and fertility in cattle following ovulation of the first-wave follicle with human chorionic gonadotropin or an agonist of gonadotropin-releasing hormone. *J. Anim. Sci.* **74**, 1074-1083.
- Schwartzman RA and Cidlowski JA. (1993). Apoptosis: The biochemistry and molecular biology of programmed cell death. *Endocr. Rev.* **14**, 133-151.
- Seidal G and Niswender G. (1980). Control of folliculogenesis and ovulation in domestic animals: puberal and adult function. In *IX International Congress of Animal Reproduction*, Spain.
- Senatore EM, Butler WR and Oltenacu PA. (1996). Relationships between energy balance and post-partum ovarian activity and fertility in first lactation dairy cows. *Anim. Sci.* **62**, 17-23.
- Short RE and Adams DC. (1988). Nutritional and hormonal interrelationships in beef cattle reproduction. *Can. J. Anim. Sci.* **68**, 29-39.
- Simmen FA. (1991). Expression of the insulin-like growth factor-1 gene and its products: complex regulation by tissue specific and hormonal factors. *Dom. Anim. Endocrinol.* **8**, 165-178.
- Simpson RB, Chase CC, Spicer LJ, Vernon RK, Hammond AC and Rae DO. (1994). Effect of exogenous insulin on plasma and follicular insulin-like growth factor I, insulin-like growth factor binding protein affinity, follicular oestradiol and progesterone, and follicular growth in superovulated Angus and Brahman cows. *J. Reprod. Fertil.* **102**, 483-492.
- Sirois J and Fortune JE. (1988). Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. *Biol. Reprod.* **39**, 308-317.
- Sirotkin AV and Nitray J. (1994). Growth hormone and prolactin affect oxytocin, vasopresin, progesterone and cyclic nucleotide secretion by bovine granulosa cells in vitro. *J. Endocrinol.* **143**, 417-422.
- Smith MF, Gutierrez CG, Armstrong DG and Webb R. (1997). Effect of LH on secretion of matrix metalloproteinases by bovine thecal cells. *J. Reprod. Fertil. Abstr. Series.* **19**.

- Souza CJH, Campbell BK and Baird DT. (1996). Follicular dynamics and ovarian steroid secretion in sheep during anoestrus. *J. Reprod. Fertil.* **108**, 101-106.
- Spears N, Boland NI, Murray AA and Gosden RG. (1994). Mouse oocyte derived from in vitro grown primary ovarian follicles are fertile. *Human Reprod.* **9**, 527-532.
- Spears N, Bruin Jpd and Gosden RG. (1996). The establishment of follicular dominance in co-cultured mouse ovarian follicles. *J. Reprod. Fertil.* **106**, 1-6.
- Spicer LJ, Echternkamp SE, Canning SF and Hammond JM. (1988). Relationship between concentration of immunoreactive insulin-like growth factor-I in follicular fluid and various biochemical markers of differentiation in bovine antral follicles. *Biol. Reprod.* **39**, 573-580.
- Spicer LJ, Enright WJ, Murphy MG and Roche JF. (1991). Effect of dietary intake on concentrations of insulin-like growth factor-I in plasma and follicular fluid, and ovarian function in heifers. *Dom. Anim. Endocrinol.* **8**, 431-437.
- Spicer LJ, Klindt J, Buonomo FC, Maurer R, Yen JT and Echternkamp SE. (1992a). Effect of porcine somatotropin on number of granulosa cell luteinizing hormone/human chorionic gonadotropin receptors, oocyte viability, and concentrations of steroids and insulin-like growth factors I and II in follicular fluid of lean and obese gilts. *J. Anim. Sci.* **70**, 3149-3157.
- Spicer L, Crowe MA, Prendiville DJ, Goulding D and Enright WJ. (1992b). Systemic but not intraovarian concentrations of insulin-like growth factor-I are affected by short-term fasting. *Biol. Reprod.* **46**, 920-925.
- Spicer LJ, Hanrahan JP, Zavy MT and Enright WJ. (1993a). Relationship between ovulation rate and concentrations of insulin-like growth factor-I in plasma during the oestrous cycle in various genotypes of sheep. *J. Reprod. Fertil.* **97**, 403-409.
- Spicer LJ, Alpizar E and Echternkamp SE. (1993b). Effects of insulin, Insulin-like growth factor I, and gonadotropins on bovine granulosa cell proliferation, progesterone production, estradiol production, and (or) insulin-like growth factor I production *in vitro*. *J. Anim. Sci.* **71**, 1232-1241.
- Spicer LJ and Echternkamp SE. (1995). The ovarian insulin and insulin-like growth factor system with an emphasis on domestic animals. *Dom. Anim. Endocrinol.* **12**, 223-245.
- Spicer LJ, Stewart RE, Hamilton TD and Keefer BE. (1995). Interaction between insulin and glucose in regulating proliferation and steroidogenesis of bovine thecal cells in vitro. *J. Reprod. Fertil. Abstr. Series* **15**, Abstract 142.
- Spicer LJ and Stewart RE. (1996a). Interactions among basic fibroblast growth factor, epidermal growth factor, insulin, and insulin-like growth factor-I on cell number and steroidogenesis of bovine thecal cells: Role of IGF-I receptors. *Biol. Reprod.* **54**, 255-263.
- Spicer LJ and Stewart RE. (1996b). Interaction among bovine somatotropin, insulin, and gonadotropins on steroid production by bovine granulosa and thecal cells. *J. Dairy Sci.* **79**, 813-821.

- Spiegelman BM and Flier JS. (1996). Adipogenesis and obesity: Rounding out the big picture. *Cell* **87**, 377-389.
- Staigmiller RB, England BG, Webb R, Short RE and Bellows RA. (1982). Estrogen secretion and gonadotropin binding by individual bovine follicles during estrus. *J. Anim. Sci.* **55**, 1473-1482.
- Stewart RE, Spicer LJ, Hamilton TD and Keefer BE. (1995). Effects of insulin-like growth factor I and insulin on proliferation and on basal and luteinizing hormone-induced steroidogenesis for insulin-like growth factor I and luteinizing hormone. *J. Anim. Sci.* **73**, 3719-3731.
- Stewart RE, Spicer LJ, Hamilton TD, Keefer BE, Dawson LJ, Morgan GL and Echternkamp SE. (1996). Levels of insulin-like growth factor (IGF) binding proteins, luteinizing hormone and IGF-I receptors, and steroids in dominant follicles during the first follicular wave in cattle exhibiting regular estrus cycles. *Endocrinology* **137**, 2842-2850.
- Stock AE and Fortune JE. (1993). Ovarian follicular dominance in cattle: Relationship between prolonged growth of the ovulatory follicle and endocrine parameters. *Endocrinology* **132**, 1108-1114.
- Stumpf M, Day L, Wolfe M, Clutter A, Stotts J, Wolfe P, Kittok R and Kinder J. (1989). Effect of oestradiol on secretion of luteinizing hormone during the follicular phase of the bovine estrous cycle. *Biol. Reprod.* **40**, 91-97.
- Sunderland SJ, Crowe MA, Boland MP, Roche JF and Ireland JJ. (1994). Selection dominance and atresia of follicles during oestrous cycle of heifers. *J. Reprod. Fertil.* **101**, 547-555.
- Sutovsky P, Flechon JE, Flechon B, Motlik J, Peynot N, Chesne P and Heyman Y. (1993). Dynamic changes of gap junctions and cytoskeleton during in vitro culture of cattle oocyte cumulus complexes. *Biol. Reprod.* **49**, 1277-1287.
- Sutton JD, Hart IC, Broster WH, Elliot RJ and Schuller E. (1986). Feeding frequency for lactating cows: effects on rumen fermentation and blood metabolites and hormones. *British J. Nutr.* **56**, 181-192.
- Swanson LV. (1989). Discussion - Interactions of nutrition and reproduction. *J. Dairy Sci.* **72**, 805-814.
- Swerdlow RS, Batt RA and Bray GA. (1976). Reproductive hormonal function in the genetically obese (ob/ob) mouse. *Endocrinology* **98**, 1359-1364.
- Takagi M, Tsukihara T, Nishikata Y and Suzuki T. (1993). Relationship between progesterone and estradiol contents of follicular fluid and the morphological appearance of granulosa cells of follicles coexisting with corpora lutea in bovine ovaries. *Theriogenology* **40**, 135-147.
- Takenaka A, Mori M, Yamada S, Ohgane J, Takahashi S and Noguchi T. (1996). Nutritional regulation of gene expression of insulin-like growth factor-binding proteins and the acid-labile subunit in various tissues of rats. *J. Endocrinol.* **150**, 33-41.

- Telfer EE. (1996). The development of methods for isolation and culture of preantral follicles from bovine and porcine ovaries. *Theriogenology* **45**, 101-110.
- Thissen JP, Ketelslegers JM and Underwood LE. (1994). Nutritional Regulation of the insulin-like growth factors. *Endocr. Rev.* **15**, 80-101.
- Thomson S, Wallace AM and Cook B. (1989). A ^{125}I -radioimmunoassay for measuring androstenedione in serum and in blood-spot samples from neonates. *Clin. Chem.* **35**, 1706-1712.
- Tian XC, Berndtson AK and Fortune JE. (1995). Differentiation of bovine preovulatory follicles during the follicular phase is associated with increases in messenger ribonucleic acid for cytochrome P450 side-chain cleavage, 3β -Hydroxysteroid dehydrogenase, and P450 17α -hydroxylase, but not P450 aromatase. *Endocrinology* **136**, 5102-5110.
- Tilly JL. (1993). Ovarian follicular atresia: a model to study the mechanism of physiological cell death. *Endocr. J.* **1**, 67-72.
- Tilly JL. (1996). Apoptosis and ovarian function. *Rev. Reprod.* **1**, 162-172.
- Tisdall DJ, Watanabe K, Hudson NL, Smith P and McNatty KP. (1995). FSH receptor gene expression during ovarian follicle development in sheep. *J. Mol. Endocrinol.* **15**, 273-281.
- Turzillo A and Fortune J. (1990). Suppression of the secondary FSH surge with bovine follicular fluid is associated with delayed ovarian follicular development in heifers. *J. Reprod. Fertil.* **89**, 643-653.
- Turzillo AM and Fortune JE. (1993). Effects of suppressing plasma FSH on ovarian follicular dominance in cattle. *J. Reprod. Fertil.* **98**, 113-119.
- Ueda N and Shah SV. (1994). Apoptosis. *J. Lab. Clin. Med.* **124**, 169-177.
- Van-Dessel HJHMT, Chandrasekher Y, Yap OWS, Lee PDK, Hintz RL, Faessen GHJ, Braat DDM, Fauser BCJM and Giudice LC. (1996). Serum and follicular fluid levels of insulin-like growth factor I (IGF-I), IGF-II, and IGF-binding protein-1 and -3 during the normal menstrual cycle. *J. Clin. Endocrinol. Metabol.* **81**, 1224-1231.
- van-Wezel IL and Rodgers RJ. (1996). Morphological characterization of bovine primordial follicles and their environment in vivo. *Biol. Reprod.* **55**, 1003-1011.
- Vaux DL and Strasser A. (1996). The molecular biology of apoptosis. *Proc. Nat. Acad. Sci. USA* **93**, 2239-2244.
- Veerkamp RF, Simm G and Oldham JD. (1994). Effects of interaction between genotype and feeding system on milk production, feed intake, efficiency and body tissue mobilization in dairy cows. *Livest. Prod. Sci.* **39**, 229-241.
- Vernon RG and Peaker M. (1983). The regulation of nutrient supply within the body. In *Nutritional physiology of farm animals* (ed. Rook and Thomas), pp. 114. Longman Group Ltd., Essex.

- Vernon RK and Spicer LJ. (1994). Effects of basic fibroblast growth factor and heparin on follicle-stimulating hormone-induced steroidogenesis by bovine granulosa cells. *J. Anim. Sci.* **72**, 2696-2702.
- Voutilainen R, Franks S, Mason HD and Martikainen H. (1996). Expression of insulin-like growth factor (IGF), IGF-Binding proteins, and IGF receptor messenger ribonucleic acids in normal and polycystic ovaries. *J. Clin. Endocrinol. Metabol.* **81**, 1003-1008.
- Wade GN and Schneider JE. (1992). Metabolic fuels and reproduction in female mammals. *Neurosci. Biobehavioural Rev.* **16**, 235-272.
- Wandji SA, Srsen V, Voss AK, Eppig JJ and Fortune JE. (1996). Initiation in vitro of growth of bovine primordial follicles. *Biol. Reprod.* **55**, 942-948.
- Wang XN and Greenwald GS. (1993a). Hypophysectomy of the cyclic mouse. I. Effects on folliculogenesis, oocyte growth, and follicle-stimulating hormone and human chorionic gonadotropin receptors. *Biol. Reprod.* **48**, 585-594.
- Wang XN and Greenwald GS. (1993b). Hypophysectomy of the cyclic mouse. II. Effects of follicle-stimulating hormone (FSH) and luteinizing hormone on folliculogenesis, FSH and human chorionic gonadotropin receptors, and steroidogenesis. *Biol. Reprod.* **48**, 595-605.
- Wertz IE and Hanley MR. (1996). Diverse molecular provocation of programmed cell death. *Trends Biochem. Sci.* **21**, 359-364.
- Ward JR, Henricks DM, Jenkins TC and Bridges WC. (1992). Serum hormone and metabolite concentrations in fasted young bulls and steers. *Dom. Anim. Endocrinol.* **9**, 97-103.
- Wathes DC, Perks CM, Davis AJ and Denning-Kendall PA. (1995). Regulation of insulin-like growth factor-I and progesterone synthesis by insulin and growth hormone in the ovine ovary. *Biol. Reprod.* **53**, 882-889.
- Watson M. (1958). Staining of tissue sections for electron microscopy with heavy metals. *J. Biophys. Bio-chem. Cytol.* **4**, 475-478.
- Webb PD. (1980). Development of the adrenal cortex in the fetal sheep: an ultrastructural study. *J. Develop. Physiol.* **2**, 161-181.
- Webb R, Baxter G, McBride D, Nordblom CD and Shaw MPK. (1985). The measurement of testosterone and oestradiol-17b using iodinated tracers and incorporating an affinity chromatography extraction procedure. *J. Steroid Biochem.* **23**, 1043-1051.
- Webb R, Gong JG, Law AS and Rusbridge SM. (1992). Control of ovarian function in cattle. *J. Reprod. Fertil. Suppl.* **45**, 141-156.
- Webb R, Gong JG and Bramley TA. (1994). Role of growth hormone and intrafollicular peptides in follicle development in cattle. *Theriogenology* **41**, 25-30.

- Webster JR, Corson ID, Littlejohn RP, Stuart SK and Suttie JM. (1996). Effects of season and nutrition on growth hormone and insulin-like growth factor-I in male red deer. *Endocrinology* **137**, 698-704.
- Weigle DS and Kuijper JL. (1996). Obesity genes and the regulation of body fat content. *BioEssays* **18**, 867-874.
- Whitaker DA, Smith EJ, Rosa GOd and Kelly JM. (1993). Some effects of nutrition and management on the fertility of dairy cattle. *Vet. Rec.* **133**, 61-64.
- White DW and Tartaglia LA. (1996). Leptin and OB-R: Body weight regulation by cytokine receptor. *Cytok. Growth Fact. Rev.* **7**, 303-309.
- Wilmot I, Schnieke AE, McWhir J, Klind AJ and Campbell KHS. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature* **385**, 810-813.
- Wolff-Exalto EAd. (1982). Influence of gonadotrophins on early follicle cell development and early oocyte growth in the immature rat. *J. Reprod. Fertil.* **66**, 537-544.
- Woolliams JA, Nisbet RS and Lovendahl P. (1992). The effect of dietary protein on metabolite concentration during fasting in calves differing genetically in dairy merit. *Anim. Prod.* **54**, 175-181.
- Woolliams JA, Angus KD and Wilson SB. (1993). Endogenous pulsing and stimulated release of growth hormone in dairy calves of high or low genetic merit. *Anim. Prod.* **56**, 1-8.
- Wrathall JHM and Knight PG. (1993). Production of immunoactive inhibin by bovine granulosa cells in serum-free culture: effects of exogenous steroids and FSH. *Dom. Anim. Endocrinol.* **10**, 289-304.
- Wood SC, Glencross RG, Bleach ECL, Lovell R, Beard AJ and Knight PG. (1993) The ability of steroid-free bovine follicular-fluid to suppress FSH-secretion and delay ovulation persists in heifers actively immunized against inhibin. *J. Endocrinol.* **136**, 137-148.
- Wright IA, Rhind SM, Smith AJ and Whyte TK. (1992). Effects of body condition and estradiol on luteinizing hormone secretion in post-partum beef cows. *Dom. Anim. Endocrinol.* **9**, 305-312.
- Wyllie AH. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **284**, 555-556.
- Xing GQ, Mackenzie DDS, McCutcheon SN and Wickham BW. (1993). Pancreatic insulin responses to exogenous glucose in Friesan heifers of low or high genetic merit for milk-fat yield. *Anim. Prod.* **56**, 171-178.
- Xu Z, Garverick HA, Smith GW, Smith MF, Hamilton SA and Youngquist RS. (1995a). Expression of messenger ribonucleic acid encoding cytochrome P450 side-chain cleavage, cytochrome P450 17 α -hydroxylase, and cytochrome P450 aromatase in bovine follicles during the first follicular wave. *Endocrinology* **136**, 981-989.

- Xu Z, Garverick HA, Smith GW, Smith MF, Hamilton SA and Youngquist RS. (1995b). Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribonucleic acids in bovine follicles during the first follicular wave. *Biol. Reprod.* **53**, 951-957.
- Yambayamba ESK, Price MA and Foxcroft GR. (1996). Hormonal status, metabolic changes, and resting metabolic rate in beef heifers undergoing compensatory growth. *J. Anim. Sci.* **74**, 57-69.
- Yelich JV, Wettemann RP, Marston TT and Spicer LJ. (1996). Luteinizing hormone, growth hormone, insulin-like growth factor-I, insulin and metabolites before puberty in heifers fed to gain at two rates. *Dom. Anim. Endocrinol.* **13**, 325-338.
- Yoshimura Y, Iwashita M, Karube M, Oda T, Akiba M, Shiokawa S, Ando M, Yoshinaga A and Nakamura Y. (1994). Growth hormone stimulates follicular development by stimulating ovarian production of insulin-like growth factor-I. *Endocrinology* **135**, 887-894.
- Yoshinaga-Hirabayashi T and Osawa Y. (1994). Steroidogenic activity of atretic follicles in the cyclic hamster ovary and relation to ultrastructural observations. *Histochem.* **102**, 59-67.
- Yu WH, Kimura M, Walczewska A, Karanth S and McCann SM. (1997). Role of leptin in hypothalamic-pituitary function. *Proc. Natl. Acad. Sci. USA* **94**, 1023-1028.
- Yuan W, Lucy MC and Smith MF. (1996). Messenger ribonucleic acid for insulin-like growth factor-I and -II, insulin-like growth factor-binding protein-2, gonadotrophin receptors, and steroidogenic enzymes in porcine follicles. *Biol. Reprod.* **55**, 1045-1054.
- Zachow RJ and Magoffin DA. (1997). Direct intraovarian effects of leptin: Impairment of the synergistic action of insulin-like growth factor-I on follicle-stimulating hormone-dependent estradiol-17 β production by rat ovarian granulosa cells. *Endocrinology* **138**, 847-850.
- Zakeri Z, Bursch W, Tenniswood M and Lockshin RA. (1995). Cell death: programmed, apoptosis, necrosis or other? *Cell Death Differentiation* **2**, 87-96.
- Zeleznik AL, Ihling LL and Basset SG. (1989). Developmental expression of Ca⁺⁺/Mg⁺⁺ -dependent endonuclease activity in rat granulosa and luteal cells. *Endocrinology* **125**, 2218-2220.
- Zhou J and Bondy C. (1993). Anatomy of the human ovarian insulin-like growth factor system. *Biol. Reprod.* **48**, 247-482.
- Zhou J, Adesanya OO, Vatzias G, Hammond JM and Bondy CA. (1996). Selective expression of insulin-like growth factor system components during porcine ovary follicular selection. *Endocrinology* **137**, 4893-4901.
- Zurek E, Foxcroft GR and Kennelly JJ. (1995). Metabolic status and interval to first ovulation in postpartum dairy cows. *J. Dairy Sci.* **78**, 1909-1920.

13. APPENDIX

13.1 ASSAY BUFFERS

Assay buffers were prepared fresh using triple-distilled water produced by the Waters Milli-Q purification system (Millipore corporation, Milford, MA, USA) as diluent.

Assay buffers were made up in 0.05 M sodium phosphate buffer (PB). Phosphate buffer was prepared as a 10X concentrated stock (0.5 M PB), by dissolving 716g disodium hydrogen orthophosphate into 4 litres of water mixed with sodium hydrogen orthophosphate (78g dissolved into 1 litre) to give a pH of 7.5.

13.1.1 0.05M PHOSPHATE BUFFER SALINE (PBS). One litre of buffer was prepared by dissolving 9 g of sodium chloride, 100 ml of 10X phosphate buffer and 100 mg thimerosal.

13.1.2 GELATIN/PBS BUFFER (PHOSGEL) consisted of 9 g sodium chloride, 1 g swine skin gelatin (300 bloom), 100 ml of phosphate buffer and 100 mg thimerosal per litre. Gelatin was dissolved in 200 ml of buffer by gentle heating in a microwave before adding it to the final diluted buffer.

13.1.3 BSA/PBS BUFFER (0.1% BSA). This buffer was prepared as for the gelatin buffer, but substituting the gelatin with bovine serum albumin (BSA, fraction V, RIA grade; Sigma).

13.1.4 BSA/PBS buffer (0.5% BSA) consisted of 9 g sodium chloride, 5 g BSA, 100 ml of phosphate buffer and 100 mg thimerosal per litre.

13.1.5 Borate buffer, pH 7.8, consisted of 3.1g boric acid (FSA laboratory supplies, Loughborough), 2 g BSA and 100 mg sodium azide (BDH chemicals Ltd. Poole) per litre. The pH was adjusted to 7.8 with 1M NaOH.

13.1.6 IGF assay buffers

13.1.6.1 Assay buffer consisted of 4.14g of NaH_2PO_4 , 3.72g of EDTA, 10 ml of 2% sodium azide, 0.5 ml Tween-20 (Sigma) and 1g BSA in one litre of water. The pH was adjusted to 7.5 with 2M NaOH.

13.1.7 IGF Competitive assay buffers

This radioimmunoassay made use of two buffers with different pH values.

13.1.7.1 IGF acid buffer (pH 2.8) consisted of 2.76g of NaH_2PO_4 , 5.84g sodium chloride, 1g BSA, 100mg sodium azide and 1 ml Triton X-100 per litre. The pH was adjusted to 2.8 by adding orthophosphoric acid (Fisons, Fisons Scientific Equipment, Loughborough).

13.1.7.2 IGF neutralising buffer (pH 7.8) consisted of 200 ml of 10X phosphate buffer, 2.92 g sodium chloride, 1 g BSA, 200 mg sodium azide and 1 ml Triton X-100 made up to 1 litre.

13.2 RADIOIMMUNOASSAY PROCEDURES

13.2.1 FOLLICLE-STIMULATING HORMONE (FSH)

Circulating FSH concentrations were measured using a RIA as described by Gong *et al.* (1995). The assay was performed in 12 X 75 polypropylene tubes (LIP, Shipley) using 0.1% BSA buffer. The assay used a purified bovine FSH preparation (USDA-FSH-I-2) as standard in a range varying from 10 to 5000 pg per tube diluted in 500 µl of buffer. Unknown samples (250 µl) were diluted up to 500 µl. Rabbit anti-ovine FSH (NIDDK-anti-oFSH-1) 200 µl was added at an initial dilution of 1:40,000 and incubated for 48 h,

after which the tracer (iodinated ovine FSH: NIDDK-oFSH-I-1) was added and incubated for further 48 h. Bound and unbound hormone were separated as described earlier (see Chapter 2), using 200 μ l donkey anti-rabbit serum (DARS) diluted 1:45 and 100 μ l of normal rabbit serum (NRS) diluted 1:350. The sensitivity of the assay was 0.08 ng/ml.

13.2.2 INSULIN

Insulin concentrations were measured using a RIA described by Osmond *et al.* (1981). The assay was performed in polypropylene tubes using 0.5% BSA buffer. Standards were diluted in 500 μ l of buffer ranging from 5 to 2500 pg per tube. Unknown samples (250 μ l) were diluted up to 500 μ l, and 200 μ l of guinea pig anti-bovine insulin (Miles Laboratories, Slough) was added at an initial dilution of 1:30,000 and incubated for 48 h. The iodinated bovine insulin tracer was then added and incubated for further 48 h. Bound and unbound hormone was separated as described earlier using 100 μ l sheep anti-guinea pig serum (SAGPS) diluted 1:25 and 100 μ l of normal guinea-pig serum (NGPS) diluted 1:200. The sensitivity of the assay was 0.04 ng/ml.

13.2.3 GROWTH HORMONE (GH)

Plasma concentrations of GH were measured by RIA described by Lovendahl *et al.* (1991) using glass tubes and borate buffer. Recombinant bovine somatotropin (American Cyanamid Co., Princeton, New Jersey, USA) was used as both standard and tracer. Standards (0.2 to 200 pg per tube) and unknown samples (50 μ l) were diluted in 100 μ l of borate buffer. Specific antibody (50 μ l of guinea pig anti-bovine GH diluted 1:10,000) was added and incubated a further 48 h. Iodinated bovine GH was added and incubated for further 48 h. Bound and unbound hormone were separated as described earlier using 100 μ l sheep anti-guinea pig serum (SAGPS) diluted 1:40 and 100 μ l of normal guinea-pig serum (NGPS) diluted 1:200. The sensitivity of the assay was 1.2 ng/ml.

13.2.4 PROGESTERONE

Plasma progesterone concentrations were measured by RIA (Corrie *et al.*, 1981), modified for non-extracted samples by Law *et al.* (1992). Standards (7.8 to 1000 pg per tube) and unknown samples (50 μ l) were diluted in 500 μ l of phosgel. Iodinated label containing 1mg/ml ANS (1-anilinonaphthyl-sulphonic acid) was added, together with 200 μ l of rabbit anti-progesterone antibody (dilution 1:16,000) and incubated for 3 h at room temperature. 200 μ l DARS (diluted at 1:35) and 100 μ l of NRS (diluted 1:300) were added and bound and unbound were separated by centrifugation. The sensitivity of the assay was 0.01 ng/ml.

In the case of culture media, the RIA was carried out in plastic mini tubes (Anachem Ltd. Luton, Beds.). Progesterone concentrations in culture media were very high. For this reason the assay sensitivity was reduced by increasing the concentration of the anti-progesterone antibody to 1:5,000 (SAPU, anti-progesterone 7044x). Culture media and standards (range, 4.8 to 10,000 pg per tube) were diluted to 100 μ l in phosgel. 100 μ l of diluted antibody and 100 μ l of label were added and incubated overnight. Second antibody (DARS; diluted 1:20) and NRS (diluted 1:200) were added. Before centrifugation, tubes were prewashed with 250 μ l of buffer incorporating 1% Tween 20. Under these conditions, the sensitivity of the assay was 80 pg per tube.

13.2.5 OESTRADIOL

Oestradiol in unextracted cultured media was measured by a double antibody radio immunoassay as previously described (Webb *et al.*, 1985). The sensitivity of the assay was decreased by increasing the amount of primary antibody. The method was as described for progesterone, but using the specific rabbit anti-estradiol antibody at a dilution of 1:10,000. The sensitivity of the assay was 5 pg per tube.

13.2.6 ANDROSTENEDIONE

Androstenedione was measured by the radioimmunoassay developed by Thomson *et al.* (1989). Similar to the procedure used for the measurement of progesterone and oestradiol measurements from culture media, the androstenedione assay sensitivity was reduced by increasing the concentration of primary antibody to 1:50,000. Culture media and standards (range, 31.2 to 8,000 pg per tube) were diluted to 100 µl in phosgel. 100 µl of diluted antibody and 100 µl of label were added and incubated overnight. Second antibody (DARS; diluted 1:20) and NRS (diluted 1:200) were added. Before centrifugation, tubes were prewashed with 250 µl of buffer incorporating 1% Tween 20. Under these conditions, the sensitivity of the assay was 200 pg per tube.

13.2.7 INSULIN-LIKE GROWTH FACTOR-I (IGF-I)

Plasma IGF-I concentrations were measured by RIA described by Armstrong *et al.* (1990), with slight modifications. Standards and samples (plasma or culture-medium) were diluted in 200 µl of IGF-I normal assay buffer and incubated overnight with rabbit anti-IGF-I antibody (dilution 1:10,000), after which iodinated label was added and incubated another 24 h. 100 µl DARS (diluted 1:20) and 100 µl of NRS (diluted 1:150) was added. Assay tubes were prewashed with cold water containing 6% Polyethylene glycol (8000; Sigma) and centrifuged. The sensitivity of the assay was 0.11 ng/ml.

For culture media, a RIA was developed to measure IGF-I in unextracted samples (see Chapter 3). This assay was based on the method described by Blum *et al.* (1988), where samples were diluted in acid buffer (pH 2.8) to dissociate the IGF from the IGFBP, and interference of IGFBP was blocked with excess IGF-II (GroPep) added together with the antibody in neutralising buffer (pH 7.8).

13.3 HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC): BUFFERS AND SAMPLE PREPARATION

Buffers were prepared fresh on the day samples were run. All buffers were filtered through a 0.22 μ m Durapore membrane filter (Millipore) and sparged for 10 min with 100 ml min⁻¹ of helium per litre of buffer to degass. The pH was adjusted with concentrated hydrochloric acid to 2.5 and 0.05% of Tween-20 was added. The buffers were continuously degassed by sparging with 20 ml min⁻¹ of helium.

13.3.1 Mobile phase buffer. consisted of 0.2 mol/l acetic acid containing 0.05 mol/l trimethylamine and 0.05% Tween-20.

To prepare one litre of HPLC mobile phase buffer; add 900 ml of water, 11.45 ml glacial acetic acid, 6.63 ml 45% trimethylamine, and 3.25 ml concentrated hydrochloric acid. Then made up to 1 litre with water.

13.3.2 Sample treatment buffer consisted of 0.8 mol/l acetic acid containing 0.20 mol/l trimethylamine and 0.05% Tween-20. Add 390 ml of water, 45.8 ml glacial acetic acid, 26.5 ml 45% trimethylamine and 13 ml concentrated hydrochloric acid. pH was adjusted to 2.5 with concentrated hydrochloric acid and made up to 1 litre with water.

13.4 Sample preparation

Plasma samples were prepared as follows: In a 1 ml Eppendorf tubes, 100 μ l of sample, 400 μ l water and 150 μ l of sample treatment buffer were added. The tubes were mixed and incubated for 20 min., 600 μ l of chloroform were added, mixed thoroughly and centrifuged at 10,000g at 4°C for 10 min. The upper fat-extracted layer was recovered and filtered through a 0.45 μ m filter.

Fractions, containing IGFBPs and IGFs, were determined by elution of ¹²⁵I-IGF-I and by IGF-I radioimmunoassay. IGFBPs were eluted between 14 to 17 min and IGF-I between 18-22 min.

2.5 ml of conditioned culture media was concentrated by lyophilisation (Edwards Freeze dryer, Modulyo. Edwards High Vacuum. Crawley, England.) and then resuspended in 250 µl of water. This was acidified with 100 µl of sample treatment buffer and filtered before loading on to the column. The column was equilibrated by pumping with the mobile phase for 1 h prior to the injection of the first sample. The spectrophotometric baseline (280 nm) was allowed to stabilise before sample injection. Acidified samples were applied with an automated injector (Waters 712 WISP), and run at a flow rate of 0.5 ml/min for 30 min before the injection of the next sample. An automated sample collector was programmed to collect the relevant fractions. The recovery efficiency of ¹²⁵I-IGF-I was 100%.

13.4 WESTERN LIGAND BLOT

Polyacrylamide gel electrophoresis (PAGE)

12% homogenous SDS:PAGE (Sodium dodecyl sulphate: polyacrylamide gel electrophoresis; Biorad) was used under non-reducing conditions for the detection of IGFBPs (Hossenlopp *et al.*, 1986) from extracted and unextracted plasma and culture media samples.

13.4.1 GEL PREPARATION

13.4.1.1 Resolving gel (12% Acrylamide) consisted of 12ml of Acrylamide:Bis (37.5:1), 7.5 ml of 1.5M Tris pH 8.6 and 10.05ml of water. The solution was degassed, 0.3ml 10% SDS added, and 0.15ml 10% Ammonium persulphate and 10 ml TEMED (Sigma) added as catalysers. The gel was poured immediately after the addition of the catalyser and overlaid with water. After polymerisation of the first gel, the stacking gel was overlaid on top of the resolving gel.

13.4.1.2 Stacking gel consisted of 2.5 ml of Acrylamide:bio (37.5:1), 3.75 ml of 0.5 M Tris pH 6.8 and 8.56 ml of water. The solution was degassed and 0.15ml of 10% SDS added, followed by 0.15ml 10% ammonium persulphate and 10 ml TEMED.

Samples were spun down and precipitate removed, and the remaining sample mixed in equal parts with Laemmli's sample buffer (LSB), heated in boiling water at 100°C for 10 min, and 100 µl loaded to the gel. Samples were subjected to 80 mA through the stacking gel. When samples reached the resolving gel the current was increased to 120 mA and kept constant until the dye reached the bottom of the gel. The electrophoresis tank was filled with running buffer (15 g Tris, 72g glycine, 50ml 10%SDS made up to 5 litres) and gels were cooled with running tap water.

13.4.2 ELECTROTRANSFER

Protein transfer onto nitro-cellulose membranes was performed using a semi-dry blotter (Semiphor TE77, Hoeffer Scientific Instruments. San Francisco California) at 112 mA for 1.5 h. The gel was placed over a nitro-cellulose membrane sandwiched between two sheets of Whatman filter paper and saturated with transfer buffer (14.4 g l⁻¹ glycine, 3.03 g l⁻¹ Tris, pH 8.3, 10 ml 10% SDS and 200 ml methanol made up to 1 litre).

13.4.3 DETECTION OF IGFBP

After electrotransfer the membrane was dried at room temperature, then washed for 30 min in Tris-saline buffer (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.5 mg ml⁻¹ sodium azide) supplemented with 3% Nonidet P40, then washed twice for 1h in Tris-saline buffer with 1% BSA, and lastly washed twice for 20 min in Tris-saline buffer with 0.1% Tween 20. The nitrocellulose membrane was then incubated overnight in Tris-saline buffer containing 1% BSA, 0.1% Tween 20 and 6 X 10⁶ cpm ¹²⁵I-IGF-I. Thereafter, the membrane was washed (4 X 30 min) with Tris-saline buffer with 0.2% Nonidet P40. After allowing to dry, the blot was exposed to Kodak X-omatic film at -70°C with intensifying screens for up to 10 days.

13.5 RT-PCR BUFFERS

13.5.1 RT'ase mix consisted of 42 μ l 50 mM MgCl_2 , 42 μ l 10 mM dNTPmix (10mM of each dATP, dTTP, dGTP, dCTP); 84 μ l 5 X first strand buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl, 15 mM MgCl_2), 12 μ l RT'ase (25 units/ ml) and 20 μ l distilled water.

13.5.2 Dilution buffer consisted of 2 μ l 50 mM MgCl_2 , 16 μ l 5 X first strand buffer and 62 μ l distilled water.

13.5.3 PCR buffer consisted of 2.5 μ l RT'ase reaction, 1.5 μ l 10X PCR buffer (500 mM KCl and 200 mM Tris-HCl pH 8.4), 14 μ l distilled water, 1 μ l primer mix (approximately 10 pmol of the upstream and downstream primers) and 1 μ l Taq polymerase (1:5 dilution; Gibco).